IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Arne HOLMGREN et al.

Group Art Unit : 1621

Appl. No : 09/926,218
(National Stage of PCT/JP00/02076)

Examiner : Kumar

I.A. Filed : March 31, 2000

For : SUBSTRATE FOR THIOREDOXIN REDUCTASE

APPEAL BRIEF UNDER 37 C.F.R. § 41.37

Commissioner for Patents
U.S. Patent and Trademark Office
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Randolph Building
401 Dulany Street
Alexandria, VA 22314

Sir :

This appeal is under 35 U.S.C. 134 from the decision of the Examiner finally rejecting claims 13-19 and 26-28 as set forth in the Final Office Action of February 26, 2007.

A Notice of Appeal to the February 26, 2007 Final Office Action was filed August 24, 2007 accompanied by a Request for Extension of Time for three months, whereby the initial due date for filing of the Appeal Brief is set to expire two months thereafter, or October 24, 2007.

Appellant hereby requests an extension of time for five months to extend the time for filing of the Appeal Brief for five months until March 24, 2008, and are concurrently filing a formal Request for Extension of Time for five months accompanied by the government fee. If for any reason the formal Request for Extension of Time is not associated with the fee and/or any extension of time is needed, this is an express request for any extension of time necessary to maintain the

pendency of the application, and authorization to charge any required extension of time fee and/or any fee necessary to maintain the pendency of the application to Deposit Account No. 19-0089.

The requisite fee under 37 C.F.R. 41.20(b)(2) in the amount of $510.00 for the filing of the Appeal Brief is being paid by check, submitted herewith.

As noted above, if for any reason any extension of time and/or any fee is required to maintain the pendency of the application, including any extension of time and/or appeal fee, authorization is hereby provided to charge any required fee, including any fee for the Appeal Brief and any necessary extension of time fee to Deposit Account No. 19-0089.

(I) REAL PARTY IN INTEREST

The real party in interest is Arne Holmgen of Stockholm, Sweden by an assignment from Daiichi Pharmaceutical Co., Ltd. recorded April 5, 2007, at Reel 019123, Frame 0190 (3 pages).

(II) RELATED APPEALS AND INTERFERENCES

Appellant notes that a previous final rejection of the Examiner was considered by the Board of Appeals with the Examiner's final rejection being reversed in the decision of the Board of Appeals on July 29, 2005.

(III) STATUS OF CLAIMS

The status of the claims is as follows:

Claims 1-12 and 20-25 are canceled, and claims 13-19 and 26-28 are pending in this application.

Of the pending claims, claims 13-19 and 26-28 have been finally rejected in the Final Office Action mailed February 26, 2007.

(IV) STATUS OF AMENDMENTS

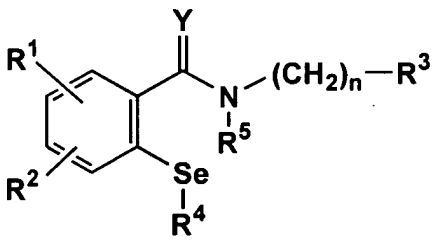
Appellant filed an Amendment Under 37 C.F.R. 1.116 on May 25, 2007. In the Amendment, claim 16 was amended to change the dependency of claim 16 from claim 17 to claim 15.

An Advisory Action was mailed June 12, 2007 indicating that Appellant's Amendment filed May 25, 2007 will be entered for purposes of appeal, whereby the claims under appeal are the claims as presented in the Amendment Under 37 C.F.R. 1.116, filed May 27, 2007. Accordingly, the Appendix herein includes claim 16 as amended in the May 25, 2007 Amendment.

(V) SUMMARY OF THE CLAIMED SUBJECT MATTER

The following description is made with respect to the independent claims and includes references to particular parts of the specification. As such, the following is merely exemplary and is not a surrender of other aspects of the present invention that are also enabled by the present specification and that are directed to equivalent structures or methods within the scope of the claims.

Independent claim 13 recites, such as disclosed in Appellant's specification at page 2, line 7 to page 3, line 9, and Figs. 1 and 2, a method for reduction of a substrate with thioredoxin reductase, comprising combining the thioredoxin reductase, the substrate and NADPH *in vitro* under conditions to reduce the substrate, the substrate comprising a substance selected from the group consisting of a compound represented by the following general formula (1) or (1') and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof:



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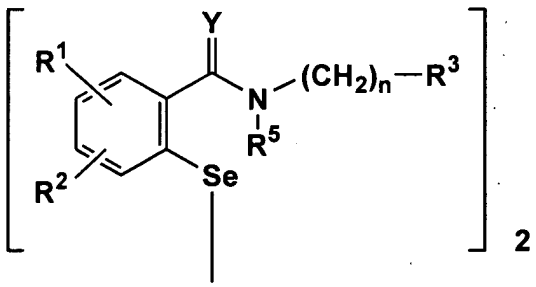
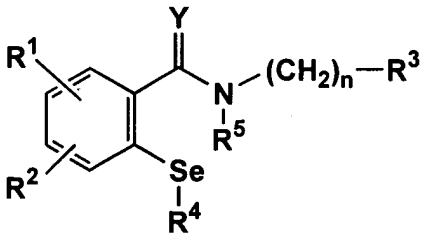


(1')

wherein R^1 and R^2 independently represent a hydrogen atom, a halogen atom, a trifluoromethyl group, a nitro group, a C_1-C_6 alkyl group, or a C_1-C_6 alkoxyl group, or R^1 and R^2 may combine together to represent methylenedioxy group; R^3 represents an aryl group, an aromatic heterocyclic group, a 5- to 7-membered cycloalkyl group, or a 5- to 7-membered cycloalkenyl group, and the aryl group, the aromatic heterocyclic group, the cycloalkyl group, and the cycloalkenyl group may be substituted with one or more substituents; R^4 represents a hydrogen atom, a hydroxyl group, a -S-glutathione group, a -S- α-amino acid group, or an aralkyl group whose aryl moiety may be substituted with one or more substituents; R^5 represents a hydrogen atom or a C_1-C_6 alkyl group, or R^4 and R^5 may combine together to represent single bond; Y represents oxygen atom or sulfur atom; n represents an integer of from 0 to 5; and the selenium atom may be oxidized.

Moreover, as disclosed in Appellant's specification, page 3, beginning in the second full paragraph (at line 10) and Fig. 3, the present invention provides as recited in independent claim 15, a method of enhancing peroxidase activity of thioredoxin reductase, comprising combining NAPDH, thioredoxin reductase, thioredoxin and a substrate *in vitro* under conditions to enhance peroxidase activity of thioredoxin reductase, the substrate comprising a substance selected from the group consisting of a compound represented by the following general formula (1) or (1') and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof:



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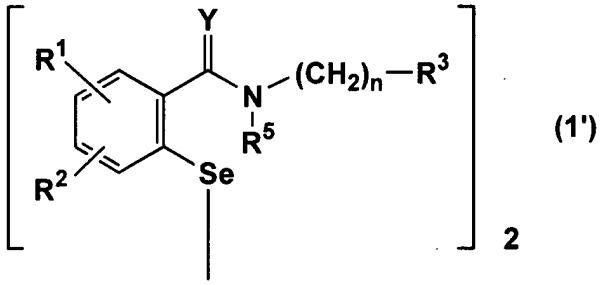
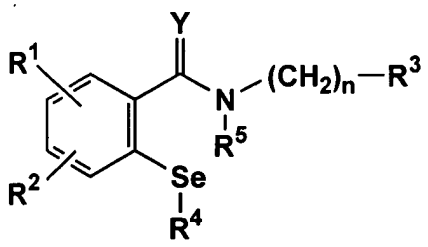


(1')

wherein R^1 and R^2 independently represent a hydrogen atom, a halogen atom, a trifluoromethyl group, a nitro group, a C_1-C_6 alkyl group, or a C_1-C_6 alkoxyl group, or R^1 and R^2 may combine together to represent methylenedioxy group; R^3 represents an aryl group, an aromatic heterocyclic group, a 5- to 7-membered cycloalkyl group, or a 5- to 7-membered cycloalkenyl group, and the aryl group, the aromatic heterocyclic group, the cycloalkyl group, and the cycloalkenyl group may be substituted with one or more substituents; R^4 represents a hydrogen atom, a hydroxyl group, a -S-glutathione group, a -S- α-amino acid group, or an aralkyl group whose aryl moiety may be substituted with one or more substituents; R^5 represents a hydrogen atom or a C_1-C_6 alkyl group, or R^4 and R^5 may combine together to represent single bond; Y represents oxygen atom or sulfur atom; n represents an integer of from 0 to 5; and the selenium atom may be oxidized.

Still further, as defined in independent claim 17, the present invention provides a method of oxidizing reduced thioredoxin by a substrate, the method comprising combining reduced thioredoxin and a substrate *in vitro* under conditions to oxidize the reduced thioredoxin with the substrate, the

substrate comprising a substance selected from the group consisting of a compound represented by the following general formula (1) or (1') and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof:



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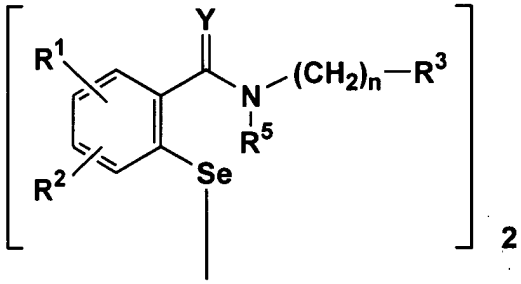
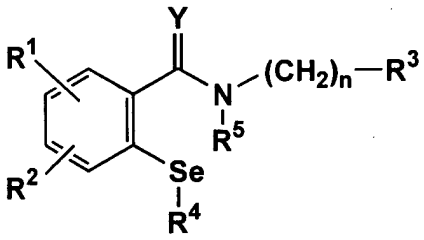


(1')

wherein R^1 and R^2 independently represent a hydrogen atom, a halogen atom, a trifluoromethyl group, a nitro group, a C_1-C_6 alkyl group, or a C_1-C_6 alkoxyl group, or R^1 and R^2 may combine together to represent methylenedioxy group; R^3 represents an aryl group, an aromatic heterocyclic group, a 5- to 7-membered cycloalkyl group, or a 5- to 7-membered cycloalkenyl group, and the aryl group, the aromatic heterocyclic group, the cycloalkyl group, and the cycloalkenyl group may be substituted with one or more substituents; R^4 represents a hydrogen atom, a hydroxyl group, a -S-glutathione group, a -S- α-amino acid group, or an aralkyl group whose aryl moiety may be substituted with one or more substituents; R^5 represents a hydrogen atom or a C_1-C_6 alkyl group, or R^4 and R^5 may combine together to represent single bond; Y represents oxygen atom or sulfur atom;

n represents an integer of from 0 to 5; and the selenium atom may be oxidized., such as disclosed in Appellant's specification at page 8, the first full paragraph (lines 2-11).

Still further, as recited in independent claim 18, the present invention provides a method for reducing a peroxide comprising combining thioredoxin, thioredoxin reductase, NAPDH and a substrate *in vitro* under conditions to reduce the peroxide, the substrate comprising a substance selected from the group consisting of a compound represented by the following general formula (1) or (1') and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof:



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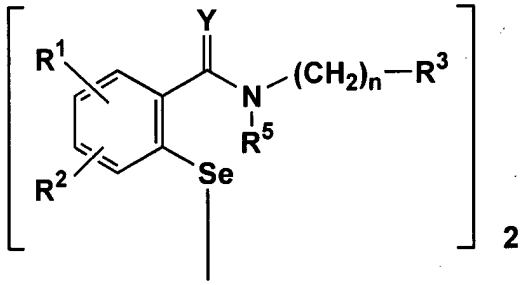
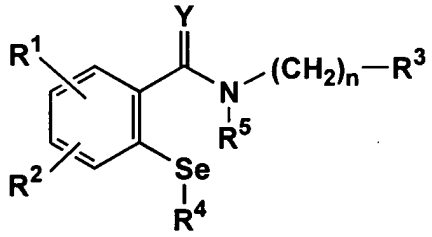


(1')

wherein R^1 and R^2 independently represent a hydrogen atom, a halogen atom, a trifluoromethyl group, a nitro group, a C_1-C_6 alkyl group, or a C_1-C_6 alkoxyl group, or R^1 and R^2 may combine together to represent methylenedioxy group; R^3 represents an aryl group, an aromatic heterocyclic group, a 5- to 7-membered cycloalkyl group, or a 5- to 7-membered cycloalkenyl group, and the aryl group, the aromatic heterocyclic group, the cycloalkyl group, and the cycloalkenyl group may be substituted with one or more substituents; R^4 represents a

hydrogen atom, a hydroxyl group, a -S-glutathione group, a -S- α-amino acid group, or an aralkyl group whose aryl moiety may be substituted with one or more substituents; R^5 represents a hydrogen atom or a C_1-C_6 alkyl group, or R^4 and R^5 may combine together to represent single bond; Y represents oxygen atom or sulfur atom; n represents an integer of from 0 to 5; and the selenium atom may be oxidized, such as disclosed in the specification at page 8, the first full paragraph (lines 2-11) and Fig. 5.

Still further, as recited in independent claim 19, the present invention provides a method of preventing peroxidation of a substance comprising combining thioredoxin, thioredoxin reductase and NADPH with a substrate *in vitro* under conditions to prevent peroxidation of the substance , the substrate being selected from the group consisting of a compound represented by the following general formula (1) or (1') and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof:



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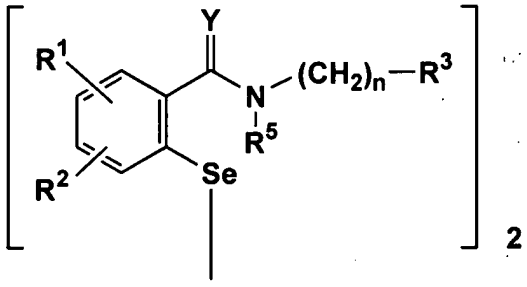


(1')

wherein R^1 and R^2 independently represent a hydrogen atom, a halogen atom, a trifluoromethyl group, a nitro group, a C_1-C_6 alkyl group, or a C_1-C_6 alkoxyl group, or R^1 and R^2 may combine together to represent methylenedioxy group; R^3 represents an aryl group, an aromatic heterocyclic group, a 5- to 7-membered cycloalkyl group, or a 5- to 7-membered cycloalkenyl group, and the aryl group, the aromatic heterocyclic group, the cycloalkyl group, and the cycloalkenyl group may be substituted with one or more substituents; R^4 represents a hydrogen atom, a hydroxyl group, a -S-glutathione group, a -S- α-amino acid group, or an aralkyl group whose aryl moiety may be substituted with one or more substituents; R^5 represents a hydrogen atom or a C_1-C_6 alkyl group, or R^4 and R^5 may combine together to represent single bond; Y represents oxygen atom or sulfur atom; n represents an integer of from 0 to 5; and the selenium atom may be oxidized., such as disclosed in the specification at page 8, the first full paragraph (lines 2-11).

(VI) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

(a) Claims 13-19 and 26-28 are rejected under 35 U.S.C. 103(a) as being unpatentable over U.S. Patent No. 4,418,069 to Welter et al. (hereinafter "Welter"), or U.S. Patent No. 4,730,053 to Dereu et al. (hereinafter "Dereu"), or EP 0 366 990 (hereinafter "EP '990"), or CA 02276984 (hereinafter "CA '984"), or WO 97/26968 (hereinafter WO '986).

(b) Claims 13-19 and 26-28 are rejected under 35 U.S.C. 103(a) as being unpatentable over the combined teachings of Arteel et al., Chem. Res. Toxicol, 1999, 12, 264-269 (hereinafter "Arteel"), Bjornstedt et al., Journal of Biological Chemistry, Vol. 270, No. 20, Issue of May 19, pp. 11761-11764, 1995 (hereinafter "Bjornstedt") , and Kumar et al., Eur. J. Biochem, 207, 435-439, 1992 (hereinafter "Kumar") and in view of Muller et al., Biochemical Pharmacology, Vol. 33, No. 20, pp. 3235-3239, 1984 (hereinafter "Muller") and Schewe, Gen. Pharmac., Vol. 26, No. 6, pp. 1153-1169, 1995(hereinafter "Schewe").

(VII) ARGUMENT

(a) Traversal of rejections of claims 13-19 and 26-28 under 35 U.S.C. 103(a) as being unpatentable over Welter (U.S. Patent No. 4,418,069), or Dereu (U.S. Patent No. 4,730,053), or EP '990 (EP 0 366 990), or CA '984 (CA 02276984), or WO '968 (WO 97/26968).

(A) Arguments for Claims 13 and 14

The rejections of claims 13 and 14 under 35 U.S.C. 103(a) as being unpatentable over Welter, or Dereu, or EP '990 or CA '984, or WO '968 are in error, the decision of the Examiner to finally reject these claims should be reversed, and the application should be remanded to the Examiner.

Appellant's independent claim 13 is directed to a method for reduction of a substrate with thioredoxin reductase, comprising combining the thioredoxin reductase, the substrate and NADPH *in vitro* under conditions to reduce the substrate, the substrate comprising a substance selected from the group consisting of a compound represented by the general formulas recited in claim 13 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Claim 14 further recites that the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one (also referred to as ebselen) or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

The rejections over each of Welter, Dereu, EP '990, CA '984 and WO '968 contend that:

> As explained in the previous office action, all these articles expressly teach administration of structurally similar compounds in vivo to achieve the same effect as claimed here in–vitro. Inasmuch as the in-vivo administration is taught achieving reduction of the substrate, it is expected that in vitro effect will be achieved in the same way, absent evidence to the contrary.

In response to these grounds of rejection, Appellant notes that the rejections are without appropriate basis in that the rejections improperly utilize Appellant's disclosure, and not the prior art, as the supporting reasoning for the rejections. The rejections contend that the documents disclose that, "the in-vivo administration is taught achieving reduction of the substrate"; however, the rejections do not point to any disclosure in any of the documents that discloses reduction of the substrate.

A review of the documents utilized in the rejections reveals that the documents disclose:

Welter - benzisoselenazolones and the *in vivo* treatment of rheumatic and arthritic diseases using benzisoselenazolones;

Dereu - S-(Carbamoyl-phenylselenyl) derivatives of mercaptanes of general formula (I), and to an *in vivo* process for the treatment of diseases caused by cell injury due to the increased formation of active oxygen metabolites;

EP '990 - stable parenteral solutions of 2-phenyl-1,2-benzisoselenazol-3(2H)-one with a discussion of uses of ebselen in treatment of numerous diseases, such as the prophylaxis and therapy of infection diseases, the therapy of malignant tumors, therapy of

rheumatic diseases, therapy of deficiencies caused by oxidative stress, and the topical treatment of inflammatory and allergic skin diseases such as psoriasis;

CA '984 - a cyclooxygenase-2-inhibitor and therapy and/or prophylaxis of such diseases which are caused in a disturbance and/or in an influence of the cyclooxygenase –2-inhibition with a peroral administration; and

WO '968 - treatment of asthma by administering to a mammal 2-phenyl-1,2-benzoisoselenazole-3(2H)-one or a pharmaceutically acceptable salt thereof.

There is no teaching in any of the documents utilized in the rejections of achieving reduction of a substrate. This is part of Appellant's disclosure, and cannot be utilized to support a rejection. The rejections must establish that one having ordinary skill in the art, from knowledge within the prior art, would have arrived at the subject matter recited in Appellant's claims.

Appellant further submits that ebselen is expected to be an inhibitor of thioredoxin reductase. In this regard, the Examiner's attention was directed to and the Board's attention is directed to Engman et al., Anticancer Res. 1997 Nov-Dec;17(6D):4599-605 (Abstract) (hereinafter "Engman"), and Arteel (which Arteel document is used in the rejection of Appellant's claims that will be argued below). From a review of Engman and Arteel, it can be seen that Engman as well as Arteel do not disclose the use of ebselen as a substrate for thioredoxin reductase. Appellant notes that Arteel (in its results and discussion, at page 268, right column, at the top of the column), cites Engman (Reference No. 22) for its disclosure of **ebselen being an inhibitor of thioredoxin reductase**, and discusses a mechanism that is not in conformance with that of Appellant's disclosed and claimed subject matter.

Thus, the prior art shows ebselen as being an inhibitor and not a substrate, which is in contrast with the Examiner's supporting assertion for the rejections. There is no reason why one having ordinary skill in the art would arrive at a method for reduction of a substrate with thioredoxin reductase, comprising combining the thioredoxin reductase, the substrate and NADPH *in vitro* under conditions to reduce the substrate, the substrate comprising a substance selected from the group consisting of a compound represented by the general formulas recited in claim 13 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Further, the rejections of record do not establish that one having ordinary skill in the art would arrive at Appellant's recited method in claim 14 for reduction of a substrate with thioredoxin reductase, comprising combining the thioredoxin reductase, the substrate and NADPH *in vitro* under conditions to reduce the substrate, the substrate comprising a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Therefore, the obviousness rejections based upon each of Welter, or Dereu, or EP '990, or CA '984, or WO '968 are without appropriate basis, and should be withdrawn.

Accordingly, the obviousness rejections based upon Welter, or Dereu, or EP '990, or CA '984, or WO '968 should be withdrawn.

(B) Arguments for Claims 15 and 16

The rejections of claims 15 and 16 under 35 U.S.C. 103(a) as being unpatentable over Welter, or Dereu, or EP '990 or CA '984, or WO '968 are in error, the decision of the Examiner to finally reject these claims should be reversed, and the application should be remanded to the Examiner.

Appellant's independent claim 15 is directed to a method of enhancing peroxidase activity of thioredoxin reductase, comprising combining NAPDH, thioredoxin reductase, thioredoxin and a substrate *in vitro* under conditions to enhance peroxidase activity of thioredoxin reductase, the substrate comprising a substance selected from the group consisting of a compound represented by the general formulas recited in claim 15 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Claim 16 further recites that the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one (also referred to as ebselen) or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

The rejections over each of Welter, Dereu, EP '990, CA '984 and WO '968 contend that:

> As explained in the previous office action, all these articles expressly teach administration of structurally similar compounds in vivo to achieve the same effect as claimed here in–vitro. Inasmuch as the in-vivo administration is taught achieving reduction of the substrate, it is expected that in vitro effect will be achieved in the same way, absent evidence to the contrary.

In response to these grounds of rejection, Appellant notes that the rejections are without appropriate basis in that the rejections improperly utilize Appellant's disclosure, and

not the prior art, as the supporting reasoning for the rejections. The rejections contend that the documents disclose that, "the in-vivo administration is taught achieving reduction of the substrate". However, the rejections do not point to any disclosure in any of the documents that discloses reduction of the substrate. Moreover, the rejections do not indicate how this assertion relates to a method of enhancing peroxidase activity of thioredoxin reductase, comprising combining NAPDH, thioredoxin reductase, thioredoxin and a substrate *in vitro* under conditions to enhance peroxidase activity of thioredoxin reductase, the substrate comprising a substance selected from the group consisting of a compound represented by the general formulas recited in claim 15 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof, or as recited in claim 16 wherein the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

As previously pointed out by Appellant, a review of the documents utilized in the rejections reveals that the documents disclose:

Welter - benzisoselenazolones and the *in vivo* treatment of rheumatic and arthritic diseases using benzisoselenazolones;

Dereu - S-(Carbamoyl-phenylselenyl) derivatives of mercaptanes of general formula (I), and to an *in vivo* process for the treatment of diseases caused by cell injury due to the increased formation of active oxygen metabolites;

EP '990 - stable parenteral solutions of 2-phenyl-1,2-benzisoselenazol-3(2H)-one with a discussion of uses of ebselen in treatment of numerous diseases, such as the

prophylaxis and therapy of infection diseases, the therapy of malignant tumors, therapy of rheumatic diseases, therapy of deficiencies caused by oxidative stress, an the topical treatment of inflammatory and allergic skin diseases such as psoriasis;

CA '984 - a cyclooxygenase-2-inhibitor and therapy and/or prophylaxis of such diseases which are caused in a disturbance and/or in an influence of the cyclooxygenase –2-inhibition with a peroral administration; and

WO '968 - treatment of asthma by administering to a mammal 2-phenyl-1,2-benzoisoselenazole-3(2H)-one or a pharmaceutically acceptable salt thereof.

There is no teaching in any of the documents utilized in the rejections of enhancing peroxidase activity of thioredoxin reductase, comprising combining NAPDH, thioredoxin reductase, thioredoxin and a substrate *in vitro* under conditions to enhance peroxidase activity of thioredoxin reductase. This is part of Appellant's disclosure, and cannot be utilized to support a rejection. The rejections must establish that one having ordinary skill in the art, from knowledge within the prior art, would have arrived at the subject matter recited in Appellant's claims.

Appellant further submits that ebselen is expected to be an inhibitor of thioredoxin reductase for the reasons set forth with respect to claims 13 and 14 above. Thus, the prior art shows ebselen as being an inhibitor and not a substrate, which is in contrast with the Examiner's supporting assertion for the rejections. There is no reason why one having ordinary skill in the art would arrive at a method of enhancing peroxidase activity of thioredoxin reductase, comprising combining NAPDH, thioredoxin reductase, thioredoxin and a substrate *in vitro* under conditions to enhance peroxidase activity of thioredoxin

reductase, the substrate comprising a substance selected from the group consisting of a compound represented by the general formulas recited in claim 15 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof, or as recited in claim 16 wherein the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Therefore, the obviousness rejections based upon each of Welter, or Dereu, or EP '990, or CA '984, or WO '968 are without appropriate basis, and should be withdrawn.

Accordingly, the obviousness rejections based upon Welter, or Dereu, or EP '990, or CA '984, or WO '968 should be withdrawn.

(C) Arguments for Claims 17 and 26

The rejections of claims 17 and 26 under 35 U.S.C. 103(a) as being unpatentable over Welter, or Dereu, or EP '990 or CA '984, or WO '968 are in error, the decision of the Examiner to finally reject these claims should be reversed, and the application should be remanded to the Examiner.

Appellant's independent claim 17 is directed to a method of oxidizing reduced thioredoxin by a substrate, the method comprising combining reduced thioredoxin and a substrate *in vitro* under conditions to oxidize the reduced thioredoxin with the substrate, the substrate comprising a substance selected from the group consisting of a compound represented by the general formulas recited in claim 17 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Claim 26 further recites that the

substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one (also referred to as ebselen) or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

The rejections over each of Welter, Dereu, EP '990, CA '984 and WO '968 contend that:

> As explained in the previous office action, all these articles expressly teach administration of structurally similar compounds in vivo to achieve the same effect as claimed here in–vitro. Inasmuch as the in-vivo administration is taught achieving reduction of the substrate, it is expected that in vitro effect will be achieved in the same way, absent evidence to the contrary.

In response to these grounds of rejection, Appellant notes that the rejections are without appropriate basis in that the rejections improperly utilize Appellant's disclosure, and not the prior art, as the supporting reasoning for the rejection. The rejections contend that the documents disclose that, "the in-vivo administration is taught achieving reduction of the substrate". However, the rejections do not point to any disclosure in any of the documents that discloses reduction of the substrate. Moreover, the rejections do not indicate how this assertion relates to a method of oxidizing reduced thioredoxin by a substrate, the method comprising combining reduced thioredoxin and a substrate *in vitro* under conditions to oxidize the reduced thioredoxin with the substrate, and a hydrate thereof and a solvate thereof, or as recited in claim 26 wherein the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

As previously pointed out by Appellant, a review of the documents utilized in the rejections reveals that the documents disclose:

Welter - benzisoselenazolones and the *in vivo* treatment of rheumatic and arthritic diseases using benzisoselenazolones;

Dereu - S-(Carbamoyl-phenylselenyl) derivatives of mercaptanes of general formula (I), and to an *in vivo* process for the treatment of diseases caused by cell injury due to the increased formation of active oxygen metabolites;

EP '990 - stable parenteral solutions of 2-phenyl-1,2-benzisoselenazol-3(2H)-one with a discussion of uses of ebselen in treatment of numerous diseases, such as the prophylaxis and therapy of infection diseases, the therapy of malignant tumors, therapy of rheumatic diseases, therapy of deficiencies caused by oxidative stress, an the topical treatment of inflammatory and allergic skin diseases such as psoriasis;

CA '984 - a cyclooxygenase-2-inhibitor and therapy and/or prophylaxis of such diseases which are caused in a disturbance and/or in an influence of the cyclooxygenase –2-inhibition with a peroral administration; and

WO '968 - treatment of asthma by administering to a mammal 2-phenyl-1,2-benzoisoselenazole-3(2H)-one or a pharmaceutically acceptable salt thereof.

There is no teaching in any of the documents utilized in the rejections of enhancing peroxidase activity of thioredoxin reductase, comprising combining NAPDH, thioredoxin reductase, thioredoxin and a substrate *in vitro* under conditions to enhance peroxidase activity of thioredoxin reductase. This is part of Appellant's disclosure, and cannot be utilized to support a rejection. The rejections must establish that one having ordinary skill in

the art, from knowledge within the prior art, would have arrived at the subject matter recited in Appellant's claims.

Appellant further submits that ebselen is expected to be an inhibitor of thioredoxin reductase for the reasons set forth with respect to claims 13 and 14 above. Thus, the prior art shows ebselen as being an inhibitor and not a substrate, which is in contrast with the Examiner's supporting assertion for the rejections. There is no reason why one having ordinary skill in the art would arrive at a method of enhancing peroxidase activity of thioredoxin reductase, comprising combining NAPDH, thioredoxin reductase, thioredoxin and a substrate *in vitro* under conditions to enhance peroxidase activity of thioredoxin reductase, the substrate comprising a substance selected from the group consisting of a compound represented by the general formulas recited in claim 17 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof, or as recited in claim 26 wherein the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Therefore, the obviousness rejections based upon each of Welter, or Dereu, or EP '990, or CA '984, or WO '968 are without appropriate basis, and should be withdrawn.

Accordingly, the obviousness rejections based upon Welter, or Dereu, or EP '990, or CA '984, or WO '968 should be withdrawn.

(D) Arguments for Claims 18 and 27

The rejections of claims 18 and 27 under 35 U.S.C. 103(a) as being unpatentable over Welter, or Dereu, or EP '990 or CA '984, or WO '968 are in error, the decision of the Examiner to finally reject these claims should be reversed, and the application should be remanded to the Examiner.

Appellant's independent claim 18 is directed to method for reducing a peroxide comprising combining thioredoxin, thioredoxin reductase, NAPDH and a substrate *in vitro* under conditions to reduce the peroxide, the substrate comprising a substance selected from the group consisting of a compound represented by the general formulas recited in claim 18 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Claim 27 further recites that the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one (also referred to as ebselen) or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

The rejections over each of Welter, Dereu, EP '990, CA '984 and WO '968 contend that:

> As explained in the previous office action, all these articles expressly teach administration of structurally similar compounds in vivo to achieve the same effect as claimed here in–vitro. Inasmuch as the in-vivo administration is taught achieving reduction of the substrate, it is expected that in vitro effect will be achieved in the same way, absent evidence to the contrary.

In response to these grounds of rejection, Appellant notes that the rejections are without appropriate basis in that the rejections improperly utilize Appellant's disclosure, and not the prior art, as the supporting reasoning for the rejection. The rejections contend that the

documents disclose that, "the in-vivo administration is taught achieving reduction of the substrate". However, the rejections do not point to any disclosure in any of the documents that discloses reduction of the substrate. Moreover, the rejections do not indicate how this assertion relates to a method for reducing a peroxide comprising combining thioredoxin, thioredoxin reductase, NAPDH and a substrate *in vitro* under conditions to reduce the peroxide, the substrate comprising a substance selected from the group consisting of a compound represented by the general formulas recited in claim 18 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof, or as recited in claim 27 wherein the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

As previously pointed out by Appellant, a review of the documents utilized in the rejections reveals that the documents disclose:

Welter - benzisoselenazolones and the *in vivo* treatment of rheumatic and arthritic diseases using benzisoselenazolones;

Dereu - S-(Carbamoyl-phenylselenyl) derivatives of mercaptanes of the general formula (I), and to an *in vivo* process for the treatment of diseases caused by cell injury due to the increased formation of active oxygen metabolites;

EP '990 - stable parenteral solutions of 2-phenyl-1,2-benzisoselenazol-3(2H)-one with a discussion of uses of ebselen in treatment of numerous diseases, such as the prophylaxis and therapy of infection diseases, the therapy of malignant tumors, therapy of rheumatic diseases, therapy of deficiencies caused by oxidative stress, an the topical

treatment of inflammatory and allergic skin diseases such as psoriasis;

CA '984 - a cyclooxygenase-2-inhibitor and therapy and/or prophylaxis of such diseases which are caused in a disturbance and/or in an influence of the cyclooxygenase –2-inhibition with a peroral administration; and

WO '968 - treatment of asthma by administering to a mammal 2-phenyl-1,2-benzoisoselenazole-3(2H)-one or a pharmaceutically acceptable salt thereof.

There is no teaching in any of the documents utilized in the rejections of reducing a peroxide comprising combining thioredoxin, thioredoxin reductase, NAPDH and a substrate *in vitro* under conditions to reduce the peroxide. This is part of Appellant's disclosure, and cannot be utilized to support a rejection. The rejections must establish that one having ordinary skill in the art, from knowledge within the prior art, would have arrived at the subject matter recited in Appellant's claims.

Appellant further submits that ebselen is expected to be an inhibitor of thioredoxin reductase for the reasons set forth with respect to claims 13 and 14 above. Thus, the prior art shows ebselen as being an inhibitor and not a substrate, which is in contrast with the Examiner's supporting assertion for the rejections. There is no reason why one having ordinary skill in the art would arrive at a method for reducing a peroxide comprising combining thioredoxin, thioredoxin reductase, NAPDH and a substrate *in vitro* under conditions to reduce the peroxide, the substrate comprising a substance selected from the group consisting of a compound represented the general formulas recited in claim 18 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof, or as recited in claim 27 wherein the substrate comprises a substance selected from the group

consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Therefore, the obviousness rejections based upon each of Welter, or Dereu, or EP '990, or CA '984, or WO '968 are without appropriate basis, and should be withdrawn.

Accordingly, the obviousness rejections based upon Welter, or Dereu, or EP '990, or CA '984, or WO '968 should be withdrawn.

(E) Arguments for Claims 19 and 28

The rejections of claims 19 and 28 under 35 U.S.C. 103(a) as being unpatentable over Welter, or Dereu, or EP '990 or CA '984, or WO '968 are in error, the decision of the Examiner to finally reject these claims should be reversed, and the application should be remanded to the Examiner.

Appellant's independent claim 19 is directed to a method of preventing peroxidation of a substance comprising combining thioredoxin, thioredoxin reductase and NADPH with a substrate *in vitro* under conditions to prevent peroxidation of the substance , the substrate being selected from the group consisting of a compound represented by the general formulas recited in claim 19 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof, and a hydrate thereof and a solvate thereof. Claim 28 further recites that the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one (also referred to as ebselen) or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

The rejections over each of Welter, Dereu, EP '990, CA '984 and WO '968 contend that:

> As explained in the previous office action, all these articles expressly teach administration of structurally similar compounds in vivo to achieve the same effect as claimed here in–vitro. Inasmuch as the in-vivo administration is taught achieving reduction of the substrate, it is expected that in vitro effect will be achieved in the same way, absent evidence to the contrary.

In response to these grounds of rejection, Appellant notes that the rejections are without appropriate basis in that the rejections improperly utilize Appellant's disclosure, and not the prior art, as the supporting reasoning for the rejections. The rejections contend that the documents disclose that, "the in-vivo administration is taught achieving reduction of the substrate". However, the rejections do not point to any disclosure in any of the documents that discloses reduction of the substrate. Moreover, the rejections do not indicate how this assertion relates to a method of preventing peroxidation of a substance comprising combining thioredoxin, thioredoxin reductase and NADPH with a substrate *in vitro* under conditions to prevent peroxidation of the substance , the substrate being selected from the group consisting of a compound represented by the general formulas recited in claim 19 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof, or as recited in claim 28 wherein the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

As previously pointed out by Appellant, a review of the documents utilized in the rejections reveals that the documents disclose:

Welter - benzisoselenazolones and the *in vivo* treatment of rheumatic and arthritic

diseases using benzisoselenazolones;

Dereu - S-(Carbamoyl-phenylselenyl) derivatives of mercaptanes of the general formula (I), and to an *in vivo* process for the treatment of diseases caused by cell injury due to the increased formation of active oxygen metabolites;

EP '990 - stable parenteral solutions of 2-phenyl-1,2-benzisoselenazol-3(2H)-one with a discussion of uses of ebselen in treatment of numerous diseases, such as the prophylaxis and therapy of infection diseases, the therapy of malignant tumors, therapy of rheumatic diseases, therapy of deficiencies caused by oxidative stress, an the topical treatment of inflammatory and allergic skin diseases such as psoriasis;

CA '984 - a cyclooxygenase-2-inhibitor and therapy and/or prophylaxis of such diseases which are caused in a disturbance and/or in an influence of the cyclooxygenase –2-inhibition with a peroral administration; and

WO '968 - treatment of asthma by administering to a mammal 2-phenyl-1,2-benzoisoselenazole-3(2H)-one or a pharmaceutically acceptable salt thereof.

There is no teaching in any of the documents utilized in the rejections of reducing a peroxide comprising combining thioredoxin, thioredoxin reductase, NAPDH and a substrate *in vitro* under conditions to reduce the peroxide. This is part of Appellant's disclosure, and cannot be utilized to support a rejection. The rejections must establish that one having ordinary skill in the art, from knowledge within the prior art, would have arrived at the subject matter recited in Appellant's claims.

Appellant further submits that ebselen is expected to be an inhibitor of thioredoxin reductase for the reasons set forth with respect to claims 13 and 14 above. Thus, the prior art

shows ebselen as being an inhibitor and not a substrate, which is in contrast with the Examiner's supporting assertion for the rejections. There is no reason why one having ordinary skill in the art would arrive at a method of preventing peroxidation of a substance comprising combining thioredoxin, thioredoxin reductase and NADPH with a substrate *in vitro* under conditions to prevent peroxidation of the substance , the substrate being selected from the group consisting of a compound represented by the general formulas recited in claim 19 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof, or as recited in claim 28 wherein the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Therefore, the obviousness rejections based upon each of Welter, or Dereu, or EP '990, or CA '984, or WO '968 are without appropriate basis, and should be withdrawn.

Accordingly, the obviousness rejections based upon Welter, or Dereu, or EP '990, or CA '984, or WO '968 should be withdrawn.

(b) Traversal of rejection of claims 13-19 and 26-28 under 35 U.S.C. 103(a) as being unpatentable over 35 U.S.C. 103(a) as being unpatentable over the combined teachings of Arteel, Bjornstedt and Kumar and in view of Muller and Schewe.

(A) Arguments for Claims 13 and 14

The rejection of claims 13 and 14 under 35 U.S.C. 103(a) as being unpatentable over the combined teachings of Arteel, Bjornstedt and Kumar and in view of Muller and Schewe is in error, the decision of the Examiner to finally reject these claims should be reversed, and the application should be remanded to the Examiner.

Appellant's independent claim 13 is directed to a method for reduction of a substrate with thioredoxin reductase, comprising combining the thioredoxin reductase, the substrate and NADPH *in vitro* under conditions to reduce the substrate, the substrate comprising a substance selected from the group consisting of a compound represented by the general formulas recited in claim 13 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Claim 14 further recites that the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one (also referred to as ebselen) or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

In this ground of rejection, the Examiner contends that:

> It would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made to use the process of Arteel et al, and Bjornstedt et al, wherein in vitro experiment is expressly done using NADPH, selenocystine and thioredoxin similar to claimed herein, by using ebselen, as taught by Muller et al and Schewe, because the latter references are expressly teaching that ebselen mimics glutathione peroxidase like activities, and Kumar et al is expressly teaching that selenite is a substrate for thioredoxin reductase and NADPH, and Bjornstedt et al expressly teaching

> that selenium is an essential element to have anti oxidant property, ebselen is having glutathione peroxidase like activity in vitro, it is obvious that ebselen when reacted with thioredoxin reductase and NADPH, would result in a method similar to claimed herein, absent evidence to the contrary.

The rejection further contends that:

> Thus, as noted in the rejection, Bjornstedt does not disclose method as recited in Applicants' claims which include the recited compounds let alone teach or suggest that compounds having a structure as ebselen would be a substrate, an inhibitor or most likely not show any reactivity.

The rejection further contends referring to Bjornstedt, ".....it is selenium which is important part of the reaction and other reference are expressly teaching that selenium is active part, and Muller is teaching ebselen to be behaving like glutathione peroxidase like activity, thus suggesting similar process as claimed herein."

Still further, referring to Kumar, the rejection notes that the structure is different but contends that, "it is selenium that is important and the references are expressly teaching equivalence of selenite, ebselen having glutathione peroxidase like activity."

Regarding Arteel not using ebselen, the rejection asserts that, "it is selenium which is important and inasmuch as ebselen is taught in a similar glutathione peroxidase like activity, the method claim is obvious as a whole, absent evidence to the contrary.

In contrast to the Examiner's assertions, Appellant again points out, as argued above, that the prior art shows ebselen as being an inhibitor and not a substrate. Therefore, the assertions in the rejection that it is selenium that is important and the references expressly teaching that selenium is active part is without appropriate basis. The rejection does not address that the prior art, such as Arteel and Engman specifically disclose that ebselen is an

inhibitor. The contention set forth in the rejection that it is selenium that is important is like saying that any compound that contains carbon would be useful to cure a disease merely because one compound capable of curing the disease contains carbon.

Still further, Appellant submits that the rejection is not clear as to how the documents are being combined. The rejection does not indicate what is meant by the combined teachings of Arteel, Bjornstedt, and Kumar. Moreover, the rejection does not explain how the various disclosures can be combined, and how Appellant's process can be arrived at when Arteel discloses that ebselen is an inhibitor. Certainly, the rejection must address this issue without utilizing Appellant's disclosure to support the rejection.

Moreover, Appellant notes that, as disclosed in Bjornstedt at page 11761, right column, third full paragraph, Bjornstedt is directed to the investigation of whether thioredoxin reductase and thioredoxin can reduce lipid hydroperoxides and if low molecular weight selenium compounds could act as charge transfer catalysts. Bjornstedt discloses that this could be an important alternative pathway for the detoxification of hydroperoxides in addition to GSH-Px-mediated reduction. Thus, as noted in the rejection, Bjornstedt does not disclose methods as recited in Appellant's claims which include the recited compounds let alone teach or suggest that compounds having a structure such as ebselen would be a substrate, an inhibitor or most likely not show any reactivity. In fact, ebselen is not a substrate for a majority of the world's thioredoxin reductases found in all bacteria, plants, yeast, etc. These thioredoxin reductases do not reduce ebselen and, in fact, ebselen acts as an inhibitor.

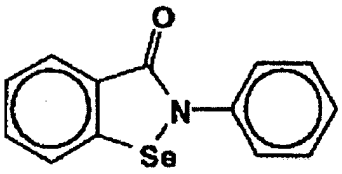
Regarding Kumar, Appellant notes that Kumar relates to the fact that sodium selenite is a redox cycling agent, and does not disclose methods as recited in Appellant's claims which include the recited compounds let alone teach or suggest that compounds having a structure such as ebselen would be a substrate, an inhibitor or most likely not show any reactivity.

Appellant submits that Arteel does not teach any methods wherein ebselen is used as a substrate for thioredoxin reductase. Arteel performs experiments with respect to the activity of mammalian thioredoxin reductase using a peroxynitrite reductase. Thus, at page 264, at the bottom of the left-hand column, Arteel discloses, "Here we investigated whether mammalian TR [thioredoxin reductase] can function as a peroxynitrite reductase." In performing the study, as disclosed in the Results section at page 265, left-hand column, Arteel infuses peroxynitrite to maintain a 0.2 pM steady-state concentration in potassium phosphate buffer. Arteel uses benzoate hydroxylation and nitrite formation as indices of oxidation reactions of peroxynitrite and of peroxynitrite reduction. Arteel particularly notes that when selenocystine or ebselen are present in the reaction mixture, there is a significant suppression of benzoate hydroxylation and an increase in nitrite formation until the NAPDH was oxidized. Arteel particularly specifies that the addition of thioredoxin did not enhance these effects (page 264, in the Abstract). Moreover, on page 265, right-hand column, Arteel discloses that. "Addition of TR to ebselen had no effect under these conditions (Y)."

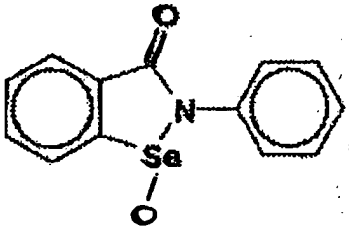
Therefore, Arteel should be considered to be nonenabling for processes wherein thioredoxin is present as Arteel does not teach or suggest any need for having the thioredoxin present in the reaction.

Appellant further notes the prior art at most teaches that ebselen is an inhibitor of thioredoxin reductase, and that ebselen selenoxide can be a substrate. However, Appellant's claimed subject matter does not include ebselen selenoxide. Arteel shows no effect of thioredoxin on reduction of ebselen selenoxide by NADPH and thioredoxin reductase.

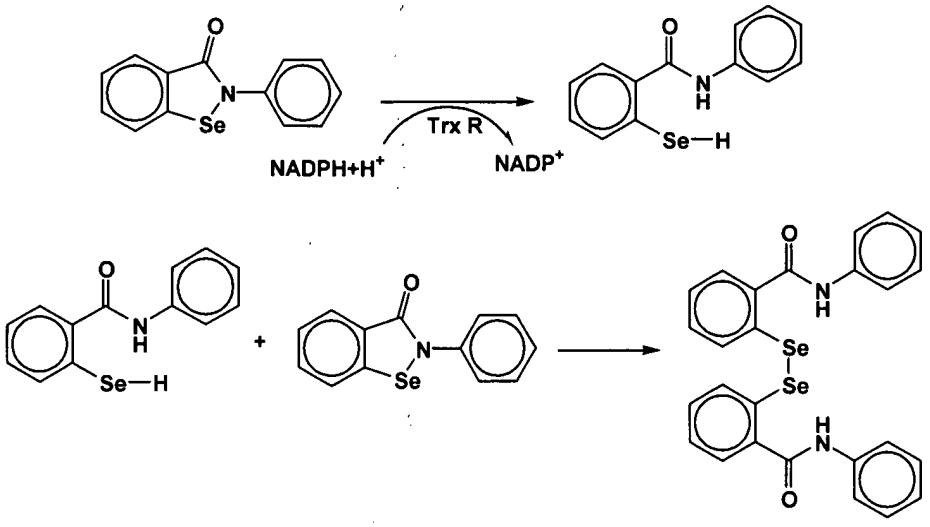
To assist a further understanding of Appellant's invention, Appellant notes that ebselen has the following formula:



Ebselen selenoxide has the following formula:



Moreover, the reaction of ebselen in Appellant's system does not form ebselen selenoxide, the compound disclosed in Arteel, but produces compounds according to the following reaction scheme, as disclosed on pages 13 and 14 of Appellant's specification.



Therefore, summarizing the above, Arteel discloses that:

(1) Ebselen inhibits thioredoxin reductase, and it is therefore expected that addition of ebselen would shut off effects of thioredoxin reductase.

(2) The strong oxidant peroxynitrite is reduced by thioredoxin reductase provided that ebselen is present. The mechanism being that ebselen is oxidized to ebselen selenoxide which is reduced by thioredoxin reductase.

(3) There is no effect by adding thioredoxin.

In contrast to the prior art utilized in the rejection, the present invention recognizes and demonstrates that ebselen is a substrate being reduced by NADPH and thioredoxin reductase with a low Km-value meaning that it is a very good substrate undergoing unlimited cycles of oxidation/reduction in the presence of hydrogen peroxide without affecting the activity of the enzyme. The reduced ebselen is called ebselen selenol and has the Se-N bond broken by reduction. The selenol is oxidized back to ebselen by hydrogen peroxide or another peroxide and a new cycle starts. The reaction is ultimately driven by NADPH. Reduced thioredoxin strongly enhances the thioredoxin reductase reaction which is also

proven by determination of the rate of reduction of ebselen by reduced thioredoxin using kinetics with tryptophan fluorescence. The result, never seen before, is that ebselen is a very efficient oxidant of reduced thioredoxin.

Thus, Appellant submits that it is by no means clear that ebselen would be a substrate for thioredoxin or thioredoxin reductase following any disclosure in Arteel, or any of the other documents utilized in the rejection. The substrate used in Arteel's work is selenocysteine, which is different from the selenocystine of Bjornstedt.

Appellant respectfully submits that one having ordinary skill in the art would not have found it obvious to have combined the disclosures to arrive at the claimed subject matter. There is no teaching or suggestion in the documents utilized in the rejection that mammalian thioredoxin reductase is a selenoenzyme and there is therefore no possibility of deducing that ebselen would be a substrate. In fact, most people testing thioredoxin reductase at the time of Appellant's invention would buy the bacterial enzyme, which was commercially available from IMCO in Stockholm as the only source and they would have seen no reactions with ebselen. Appellant's experiments utilized preparations of mammalian thioredoxin reductase from human placenta or calf thymus and with that Appellant observed that ebselen is a substrate. An extremely fast reaction with thioredoxin was part of Appellant's findings.

Muller and Schewe are utilized in the rejection for the assertion that they disclose that ebselen mimics glutathione peroxidase. While it may be true that ebselen mimics glutathione peroxidase, that is not a major concern relative to Appellant's recited methods as the issue relates to the fact that one having ordinary skill in the art at the time of Appellant' invention

would not have knowledge of ebselen being a thioredoxin reductase substrate, or a thioredoxin oxidant.

There is no supporting disclosure in the documents of record, which documents include as an author one of the coinventors of the present application, i.e., Arne Holmgren, who is a co-author of Bjornstedt and Kumar, to adequately provide a basis for the rejection.

There is no reason why one having ordinary skill in the art would arrive at a method for reduction of a substrate with thioredoxin reductase, comprising combining the thioredoxin reductase, the substrate and NADPH *in vitro* under conditions to reduce the substrate, the substrate comprising a substance selected from the group consisting of a compound represented by the general formulas recited in claim 13 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Further, the rejection does not establish that one having ordinary skill in the art would arrive at Appellant's recited method in claim 14 for reduction of a substrate with thioredoxin reductase, comprising combining the thioredoxin reductase, the substrate and NADPH *in vitro* under conditions to reduce the substrate, the substrate comprising a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Therefore, the obviousness rejection is without appropriate basis, and should be withdrawn.

Accordingly, the obviousness rejection should be withdrawn.

(B) Arguments for Claims 15 and 16

The rejection of claims 15 and 16 under 35 U.S.C. 103(a) as being unpatentable over the combined teachings of Arteel, Bjornstedt and Kumar and in view of Muller and Schewe is in error, the decision of the Examiner to finally reject these claims should be reversed, and the application should be remanded to the Examiner.

Appellant's independent claim 15 is directed to a method of enhancing peroxidase activity of thioredoxin reductase, comprising combining NAPDH, thioredoxin reductase, thioredoxin and a substrate *in vitro* under conditions to enhance peroxidase activity of thioredoxin reductase, the substrate comprising a substance selected from the group consisting of a compound represented by the general formulas recited in claim 15 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Claim 16 further recites that the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one (also referred to as ebselen) or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

In this ground of rejection, the Examiner contends that:

> It would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made to use the process of Arteel et al, and Bjornstedt et al, wherein in vitro experiment is expressly done using NADPH, selenocystine and thioredoxin similar to claimed herein, by using ebselen, as taught by Muller et al and Schewe, because the latter references are expressly teaching that ebselen mimics glutathione peroxidase like activities, and Kumar et al is expressly teaching that selenite is a substrate for thioredoxin reductase and NADPH, and Bjornstedt et al expressly teaching that selenium is an essential element to have anti oxidant property, ebselen is having glutathione peroxidase like activity in vitro, it is obvious that ebselen when reacted with thioredoxin reductase and NADPH, would result in a method similar to claimed herein, absent evidence to the contrary.

The rejection further contends that:

> Thus, as noted in the rejection, Bjornstedt does not disclose method as recited in Applicants' claims which include the recited compounds let alone teach or suggest that compounds having a structure as ebselen would be a substrate, an inhibitor or most likely not show any reactivity.

The rejection further contends referring to Bjornstedt, ".....it is selenium which is important part of the reaction and other reference are expressly teaching that selenium is active part, and Muller is teaching ebselen to be behaving like glutathione peroxidase like activity, thus suggesting similar process as claimed herein."

Still further, referring to Kumar, the rejection notes that the structure is different but contends that, "it is selenium that is important and the references are expressly teaching equivalence of selenite, ebselen having glutathione peroxidase like activity."

Regarding Arteel not using ebselen, the rejection asserts that, "it is selenium which is important and inasmuch as ebselen is taught in a similar glutathione peroxidase like activity , the method claim is obvious as a whole, absent evidence to the contrary.

In contrast to the Examiner's assertions, Appellant again points out, as argued above, that the prior art shows ebselen as being an inhibitor and not a substrate. Therefore, the assertions in the rejection that it is selenium that is important and the references expressly teaching that selenium is active part is without appropriate basis. The rejection does not address that the prior art, such as Arteel and Engman specifically disclose that ebselen is an inhibitor. The contention set forth in the rejection that it is selenium that is important is like

saying that any compound that contains carbon would be useful to cure a disease merely because one compound capable of curing the disease contains carbon.

Still further, Appellant submits that the rejection is not clear as to how the documents are being combined. The rejection does not indicate what is meant by the combined teachings of Arteel, Bjornstedt, and Kumar. Moreover, the rejection does not explain how the various disclosures can be combined, and how Appellant's process can be arrived at when Arteel discloses that ebselen is an inhibitor. Certainly, the rejection must address this issue without utilizing Appellant's disclosure to support the rejection.

Moreover, Appellant notes that, as disclosed in Bjornstedt at page 11761, right column, third full paragraph, Bjornstedt is directed to the investigation of whether thioredoxin reductase and thioredoxin can reduce lipid hydroperoxides and if low molecular weight selenium compounds could act as charge transfer catalysts. Bjornstedt discloses that this could be an important alternative pathway for the detoxification of hydroperoxides in addition to GSH-Px-mediated reduction. Thus, as noted in the rejection, Bjornstedt does not disclose methods as recited in Appellant's claims which include the recited compounds let alone teach or suggest that compounds having a structure such as ebselen would be a substrate, an inhibitor or most likely not show any reactivity. In fact, ebselen is not a substrate for a majority of the world's thioredoxin reductases found in all bacteria, plants, yeast, etc. These thioredoxin reductases do not reduce ebselen and, in fact, ebselen acts as an inhibitor.

Regarding Kumar, Appellant notes that Kumar relates to the fact that sodium selenite is a redox cycling agent, and does not disclose methods as recited in Appellant's claims

which include the recited compounds let alone teach or suggest that compounds having a structure such as ebselen would be a substrate, an inhibitor or most likely not show any reactivity.

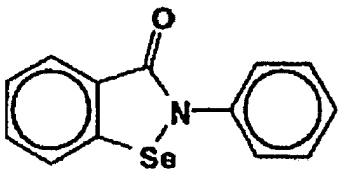
Appellant submits that Arteel does not teach any methods wherein ebselen is used as a substrate for thioredoxin reductase. Arteel performs experiments with respect to the activity of mammalian thioredoxin reductase using a peroxynitrite reductase. Thus, at page 264, at the bottom of the left-hand column, Arteel discloses, "Here we investigated whether mammalian TR [thioredoxin reductase] can function as a peroxynitrite reductase." In performing the study, as disclosed in the Results section at page 265, left-hand column, Arteel infuses peroxynitrite to maintain a 0.2 pM steady-state concentration in potassium phosphate buffer. Arteel uses benzoate hydroxylation and nitrite formation as indices of oxidation reactions of peroxynitrite and of peroxynitrite reduction. Arteel particularly notes that when selenocystine or ebselen are present in the reaction mixture, there is a significant suppression of benzoate hydroxylation and an increase in nitrite formation until the NAPDH was oxidized. Arteel particularly specifies that the addition of thioredoxin did not enhance these effects (page 264, in the Abstract). Moreover, on page 265, right-hand column, Arteel discloses that. "Addition of TR to ebselen had no effect under these conditions (Y)."

Therefore, Arteel should be considered to be nonenabling for processes wherein thioredoxin is present as Arteel does not teach or suggest any need for having the thioredoxin present in the reaction.

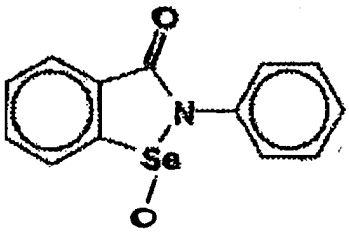
Appellant further notes the prior art at most teaches that ebselen is an inhibitor of thioredoxin reductase, and that ebselen selenoxide can be a substrate. However, Appellant's

claimed subject matter does not include ebselen selenoxide. Arteel shows no effect of thioredoxin on reduction of ebselen selenoxide by NADPH and thioredoxin reductase.

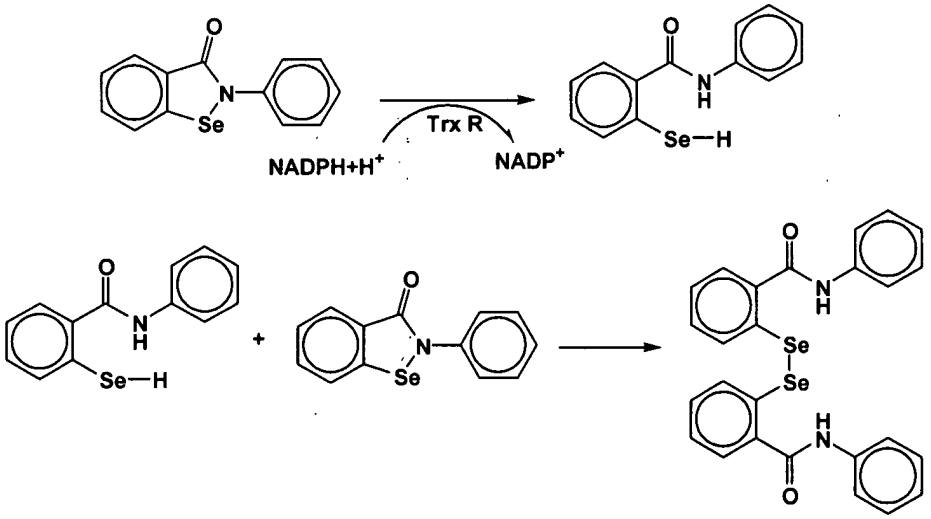
To assist a further understanding of Appellant's invention, Appellant notes that ebselen has the following formula:



Ebselen selenoxide has the following formula:



Moreover, the reaction of ebselen in Appellant's system does not form ebselen selenoxide, the compound disclosed in Arteel, but produces compounds according to the following reaction scheme, as disclosed on pages 13 and 14 of Appellant's specification.



Therefore, summarizing the above, Arteel discloses that:

(1) Ebselen inhibits thioredoxin reductase, and it is therefore expected that addition of ebselen would shut off effects of thioredoxin reductase.

(2) The strong oxidant peroxynitrite is reduced by thioredoxin reductase provided that ebselen is present. The mechanism being that ebselen is oxidized to ebselen selenoxide which is reduced by thioredoxin reductase.

(3) There is no effect by adding thioredoxin.

In contrast to the prior art utilized in the rejection, the present invention recognizes and demonstrates that ebselen is a substrate being reduced by NADPH and thioredoxin reductase with a low Km-value meaning that it is a very good substrate undergoing unlimited cycles of oxidation/reduction in the presence of hydrogen peroxide without affecting the activity of the enzyme. The reduced ebselen is called ebselen selenol and has the Se-N bond broken by reduction. The selenol is oxidized back to ebselen by hydrogen peroxide or another peroxide and a new cycle starts. The reaction is ultimately driven by NADPH. Reduced thioredoxin strongly enhances the thioredoxin reductase reaction which is also proven by determination of the rate of reduction of ebselen by reduced thioredoxin using kinetics with tryptophan fluorescence. The result, never seen before, is that ebselen is a very efficient oxidant of reduced thioredoxin.

Thus, Appellant submits that it is by no means clear that ebselen would be a substrate for thioredoxin or thioredoxin reductase following any disclosure in Arteel, or any of the other documents utilized in the rejection. The substrate used in Arteel's work is selenocysteine, which is different from the selenocystine of Bjornstedt.

Appellant respectfully submits that one having ordinary skill in the art would not have found it obvious to have combined the disclosures to arrive at the claimed subject matter. There is no teaching or suggestion in the documents utilized in the rejection that mammalian thioredoxin reductase is a selenoenzyme and there is therefore no possibility of deducing that ebselen would be a substrate. In fact, most people testing thioredoxin reductase at the time of Appellant's invention would buy the bacterial enzyme, which was commercially available from IMCO in Stockholm as the only source and they would have seen no reactions with ebselen. Appellant's experiments utilized preparations of mammalian thioredoxin reductase from human placenta or calf thymus and with that Appellant observed that ebselen is a substrate. An extremely fast reaction with thioredoxin was part of Appellant's findings.

Muller and Schewe are utilized in the rejection for the assertion that they disclose that ebselen mimics glutathione peroxidase. While it may be true that ebselen mimics glutathione peroxidase, that is not a major concern relative to Appellant's recited methods as the issue relates to the fact that one having ordinary skill in the art at the time of Appellant' invention would not have knowledge of ebselen being a thioredoxin reductase substrate, or a thioredoxin oxidant.

There is no supporting disclosure in the documents of record, which documents include as an author one of the coinventors of the present application, i.e., Arne Holmgren, who is a co-author of Bjornstedt and Kumar, to adequately provide a basis for the rejection.

There is no reason why one having ordinary skill in the art would arrive at a method of enhancing peroxidase activity of thioredoxin reductase, comprising combining NAPDH,

thioredoxin reductase, thioredoxin and a substrate *in vitro* under conditions to enhance peroxidase activity of thioredoxin reductase, the substrate comprising a substance selected from the group consisting of a compound represented by the general formulas recited in claim 15 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Further, the rejection does not establish that one having ordinary skill in the art would arrive at Appellant's recited method in claim 16 which further recites that the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one (also referred to as ebselen) or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Therefore, the obviousness rejection is without appropriate basis, and should be withdrawn.

Accordingly, the obviousness rejection should be withdrawn.

(C) Arguments for Claims 17 and 26

The rejection of claims 17 and 26 under 35 U.S.C. 103(a) as being unpatentable over the combined teachings of Arteel, Bjornstedt and Kumar and in view of Muller and Schewe is in error, the decision of the Examiner to finally reject these claims should be reversed, and the application should be remanded to the Examiner.

Appellant's independent claim 17 is directed to a method of oxidizing reduced thioredoxin by a substrate, the method comprising combining reduced thioredoxin and a substrate *in vitro* under conditions to oxidize the reduced thioredoxin with the substrate, the substrate comprising a substance selected from the group consisting of a compound represented by the general formulas recited in claim 17 and a physiologically acceptable salt

thereof, and a hydrate thereof and a solvate thereof. Claim 26 further recites that the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one (also referred to as ebselen) or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

In this ground of rejection, the Examiner contends that:

> It would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made to use the process of Arteel et al, and Bjornstedt et al, wherein in vitro experiment is expressly done using NADPH, selenocystine and thioredoxin similar to claimed herein, by using ebselen, as taught by Muller et al and Schewe, because the latter references are expressly teaching that ebselen mimics glutathione peroxidase like activities, and Kumar et al is expressly teaching that selenite is a substrate for thioredoxin reductase and NADPH, and Bjornstedt et al expressly teaching that selenium is an essential element to have anti oxidant property, ebselen is having glutathione peroxidase like activity in vitro, it is obvious that ebselen when reacted with thioredoxin reductase and NADPH, would result in a method similar to claimed herein, absent evidence to the contrary.

The rejection further contends that:

> Thus, as noted in the rejection, Bjornstedt does not disclose method as recited in Applicants' claims which include the recited compounds let alone teach or suggest that compounds having a structure as ebselen would be a substrate, an inhibitor or most likely not show any reactivity.

The rejection further contends referring to Bjornstedt, ".....it is selenium which is important part of the reaction and other reference are expressly teaching that selenium is active part, and Muller is teaching ebselen to be behaving like glutathione peroxidase like activity, thus suggesting similar process as claimed herein."

Still further, referring to Kumar, the rejection notes that the structure is different but contends that, "it is selenium that is important and the references are expressly teaching equivalence of selenite, ebselen having glutathione peroxidase like activity."

Regarding Arteel not using ebselen, the rejection asserts that, "it is selenium which is important and inasmuch as ebselen is taught in a similar glutathione peroxidase like activity, the method claim is obvious as a whole, absent evidence to the contrary.

In contrast to the Examiner's assertions, Appellant again points out, as argued above, that the prior art shows ebselen as being an inhibitor and not a substrate. Therefore, the assertions in the rejection that it is selenium that is important and the references expressly teaching that selenium is active part is without appropriate basis. The rejection does not address that the prior art, such as Arteel and Engman specifically disclose that ebselen is an inhibitor. The contention set forth in the rejection that it is selenium that is important is like saying that any compound that contains carbon would be useful to cure a disease merely because one compound capable of curing the disease contains carbon.

Still further, Appellant submits that the rejection is not clear as to how the documents are being combined. The rejection does not indicate what is meant by the combined teachings of Arteel, Bjornstedt, and Kumar. Moreover, the rejection does not explain how the various disclosures can be combined, and how Appellant's process can be arrived at when Arteel discloses that ebselen is an inhibitor. Certainly, the rejection must address this issue without utilizing Appellant's disclosure to support the rejection.

Moreover, Appellant notes that, as disclosed in Bjornstedt at page 11761, right column, third full paragraph, Bjornstedt is directed to the investigation of whether

thioredoxin reductase and thioredoxin can reduce lipid hydroperoxides and if low molecular weight selenium compounds could act as charge transfer catalysts. Bjornstedt discloses that this could be an important alternative pathway for the detoxification of hydroperoxides in addition to GSH-Px-mediated reduction. Thus, as noted in the rejection, Bjornstedt does not disclose methods as recited in Appellant's claims which include the recited compounds let alone teach or suggest that compounds having a structure such as ebselen would be a substrate, an inhibitor or most likely not show any reactivity. In fact, ebselen is not a substrate for a majority of the world's thioredoxin reductases found in all bacteria, plants, yeast, etc. These thioredoxin reductases do not reduce ebselen and, in fact, ebselen acts as an inhibitor.

Regarding Kumar, Appellant notes that Kumar relates to the fact that sodium selenite is a redox cycling agent, and does not disclose methods as recited in Appellant's claims which include the recited compounds let alone teach or suggest that compounds having a structure such as ebselen would be a substrate, an inhibitor or most likely not show any reactivity.

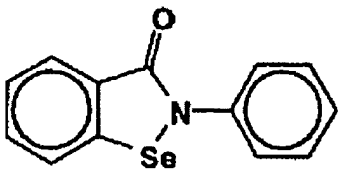
Appellant submits that Arteel does not teach any methods wherein ebselen is used as a substrate for thioredoxin reductase. Arteel performs experiments with respect to the activity of mammalian thioredoxin reductase using a peroxynitrite reductase. Thus, at page 264, at the bottom of the left-hand column, Arteel discloses, "Here we investigated whether mammalian TR [thioredoxin reductase] can function as a peroxynitrite reductase." In performing the study, as disclosed in the Results section at page 265, left-hand column, Arteel infuses peroxynitrite to maintain a 0.2 pM steady-state concentration in potassium

phosphate buffer. Arteel uses benzoate hydroxylation and nitrite formation as indices of oxidation reactions of peroxynitrite and of peroxynitrite reduction. Arteel particularly notes that when selenocystine or ebselen are present in the reaction mixture, there is a significant suppression of benzoate hydroxylation and an increase in nitrite formation until the NAPDH was oxidized. Arteel particularly specifies that the addition of thioredoxin did not enhance these effects (page 264, in the Abstract). Moreover, on page 265, right-hand column, Arteel discloses that. "Addition of TR to ebselen had no effect under these conditions (Y)."

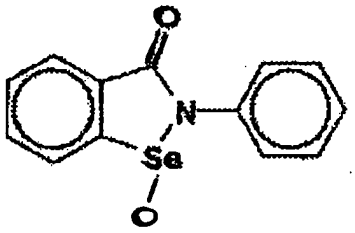
Therefore, Arteel should be considered to be nonenabling for processes wherein thioredoxin is present as Arteel does not teach or suggest any need for having the thioredoxin present in the reaction.

Appellant further notes the prior art at most teaches that ebselen is an inhibitor of thioredoxin reductase, and that ebselen selenoxide can be a substrate. However, Appellant's claimed subject matter does not include ebselen selenoxide. Arteel shows no effect of thioredoxin on reduction of ebselen selenoxide by NADPH and thioredoxin reductase.

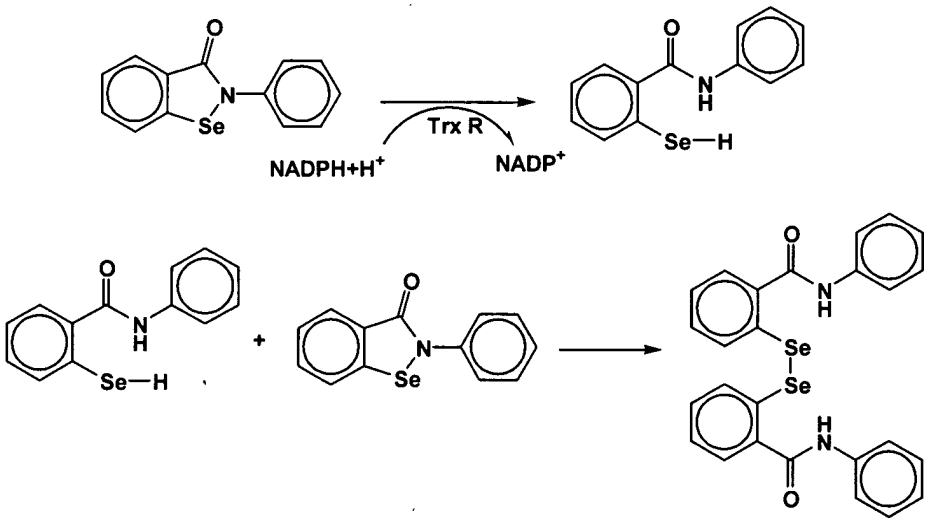
To assist a further understanding of Appellant's invention, Appellant notes that ebselen has the following formula:



Ebselen selenoxide has the following formula:



Moreover, the reaction of ebselen in Appellant's system does not form ebselen selenoxide, the compound disclosed in Arteel, but produces compounds according to the following reaction scheme, as disclosed on pages 13 and 14 of Appellant's specification.



Therefore, summarizing the above, Arteel discloses that:

(1) Ebselen inhibits thioredoxin reductase, and it is therefore expected that addition of ebselen would shut off effects of thioredoxin reductase.

(2) The strong oxidant peroxynitrite is reduced by thioredoxin reductase provided that ebselen is present. The mechanism being that ebselen is oxidized to ebselen selenoxide which is reduced by thioredoxin reductase.

(3) There is no effect by adding thioredoxin.

In contrast to the prior art utilized in the rejection, the present invention recognizes and demonstrates that ebselen is a substrate being reduced by NADPH and thioredoxin reductase with a low Km-value meaning that it is a very good substrate undergoing unlimited cycles of oxidation/reduction in the presence of hydrogen peroxide without affecting the activity of the enzyme. The reduced ebselen is called ebselen selenol and has the Se-N bond broken by reduction. The selenol is oxidized back to ebselen by hydrogen peroxide or another peroxide and a new cycle starts. The reaction is ultimately driven by NADPH. Reduced thioredoxin strongly enhances the thioredoxin reductase reaction which is also proven by determination of the rate of reduction of ebselen by reduced thioredoxin using kinetics with tryptophan fluorescence. The result, never seen before, is that ebselen is a very efficient oxidant of reduced thioredoxin.

Thus, Appellant submits that it is by no means clear that ebselen would be a substrate for thioredoxin or thioredoxin reductase following any disclosure in Arteel, or any of the other documents utilized in the rejection. The substrate used in Arteel's work is selenocysteine, which is different from the selenocystine of Bjornstedt.

Appellant respectfully submits that one having ordinary skill in the art would not have found it obvious to have combined the disclosures to arrive at the claimed subject matter. There is no teaching or suggestion in the documents utilized in the rejection that mammalian thioredoxin reductase is a selenoenzyme and there is therefore no possibility of deducing that ebselen would be a substrate. In fact, most people testing thioredoxin reductase at the time of Appellant's invention would buy the bacterial enzyme, which was

commercially available from IMCO in Stockholm as the only source and they would have seen no reactions with ebselen. Appellant's experiments utilized preparations of mammalian thioredoxin reductase from human placenta or calf thymus and with that Appellant observed that ebselen is a substrate. An extremely fast reaction with thioredoxin was part of Appellant's findings.

Muller and Schewe are utilized in the rejection for the assertion that they disclose that ebselen mimics glutathione peroxidase. While it may be true that ebselen mimics glutathione peroxidase, that is not a major concern relative to Appellant's recited methods as the issue relates to the fact that one having ordinary skill in the art at the time of Appellant' invention would not have knowledge of ebselen being a thioredoxin reductase substrate, or a thioredoxin oxidant.

There is no supporting disclosure in the documents of record, which documents include as an author one of the coinventors of the present application, i.e., Arne Holmgren, who is a co-author of Bjornstedt and Kumar, to adequately provide a basis for the rejection.

There is no reason why one having ordinary skill in the art would arrive at a method of oxidizing reduced thioredoxin by a substrate, the method comprising combining reduced thioredoxin and a substrate *in vitro* under conditions to oxidize the reduced thioredoxin with the substrate, the substrate comprising a substance selected from the group consisting of a compound represented by the general formulas recited in claim 17 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Further, the rejection does not establish that one having ordinary skill in the art would arrive at Appellant's recited method in claim 26 which further recites that the substrate comprises a substance selected

from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one (also referred to as ebselen) or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Therefore, the obviousness rejection is without appropriate basis, and should be withdrawn.

Accordingly, the obviousness rejection should be withdrawn.

(D) Arguments for Claims 18 and 27

The rejection of claims 18 and 27 under 35 U.S.C. 103(a) as being unpatentable over the combined teachings of Arteel, Bjornstedt and Kumar and in view of Muller and Schewe is in error, the decision of the Examiner to finally reject these claims should be reversed, and the application should be remanded to the Examiner.

Appellant's independent claim 18 is directed to a method for reducing a peroxide comprising combining thioredoxin, thioredoxin reductase, NAPDH and a substrate *in vitro* under conditions to reduce the peroxide, the substrate comprising a substance selected from the group consisting of a compound represented by the general formulas recited in claim 18 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Claim 27 further recites that the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one (also referred to as ebselen) or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

In this ground of rejection, the Examiner contends that:

> It would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made to use the process of Arteel et al, and

> Bjornstedt et al, wherein in vitro experiment is expressly done using NADPH, selenocystine and thioredoxin similar to claimed herein, by using ebselen, as taught by Muller et al and Schewe, because the latter references are expressly teaching that ebselen mimics glutathione peroxidase like activities, and Kumar et al is expressly teaching that selenite is a substrate for thioredoxin reductase and NADPH, and Bjornstedt et al expressly teaching that selenium is an essential element to have anti oxidant property, ebselen is having glutathione peroxidase like activity in vitro, it is obvious that ebselen when reacted with thioredoxin reductase and NADPH, would result in a method similar to claimed herein, absent evidence to the contrary.

The rejection further contends that:

> Thus, as noted in the rejection, Bjornstedt does not disclose method as recited in Applicants' claims which include the recited compounds let alone teach or suggest that compounds having a structure as ebselen would be a substrate, an inhibitor or most likely not show any reactivity.

The rejection further contends referring to Bjornstedt, ".....it is selenium which is important part of the reaction and other reference are expressly teaching that selenium is active part, and Muller is teaching ebselen to be behaving like glutathione peroxidase like activity, thus suggesting similar process as claimed herein."

Still further, referring to Kumar et al., the rejection notes that the structure is different but contends that, "it is selenium that is important and the references are expressly teaching equivalence of selenite, ebselen having glutathione peroxidase like activity."

Regarding Arteel not using ebselen, the rejection asserts that, "it is selenium which is important and inasmuch as ebselen is taught in a similar glutathione peroxidase like activity , the method claim is obvious as a whole, absent evidence to the contrary.

In contrast to the Examiner's assertions, Appellant again points out, as argued above, that the prior art shows ebselen as being an inhibitor and not a substrate. Therefore, the

assertions in the rejection that it is selenium that is important and the references expressly teaching that selenium is active part is without appropriate basis. The rejection does not address that the prior art, such as Arteel and Engman specifically disclose that ebselen is an inhibitor. The contention set forth in the rejection that it is selenium that is important is like saying that any compound that contains carbon would be useful to cure a disease merely because one compound capable of curing the disease contains carbon.

Still further, Appellant submits that the rejection is not clear as to how the documents are being combined. The rejection does not indicate what is meant by the combined teachings of Arteel, Bjornstedt, and Kumar. Moreover, the rejection does not explain how the various disclosures can be combined, and how Appellant's process can be arrived at when Arteel discloses that ebselen is an inhibitor. Certainly, the rejection must address this issue without utilizing Appellant's disclosure to support the rejection.

Moreover, Appellant notes that, as disclosed in Bjornstedt at page 11761, right column, third full paragraph, Bjornstedt is directed to the investigation of whether thioredoxin reductase and thioredoxin can reduce lipid hydroperoxides and if low molecular weight selenium compounds could act as charge transfer catalysts. Bjornstedt discloses that this could be an important alternative pathway for the detoxification of hydroperoxides in addition to GSH-Px-mediated reduction. Thus, as noted in the rejection, Bjornstedt does not disclose methods as recited in Appellant's claims which include the recited compounds let alone teach or suggest that compounds having a structure such as ebselen would be a substrate, an inhibitor or most likely not show any reactivity. In fact, ebselen is not a substrate for a majority of the world's thioredoxin

reductases found in all bacteria, plants, yeast, etc. These thioredoxin reductases do not reduce ebselen and, in fact, ebselen acts as an inhibitor.

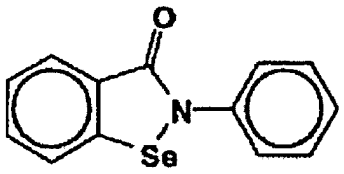
Regarding Kumar, Appellant notes that Kumar relates to the fact that sodium selenite is a redox cycling agent, and does not disclose methods as recited in Appellant's claims which include the recited compounds let alone teach or suggest that compounds having a structure such as ebselen would be a substrate, an inhibitor or most likely not show any reactivity.

Appellant submits that Arteel does not teach any methods wherein ebselen is used as a substrate for thioredoxin reductase. Arteel performs experiments with respect to the activity of mammalian thioredoxin reductase using a peroxynitrite reductase. Thus, at page 264, at the bottom of the left-hand column, Arteel discloses, "Here we investigated whether mammalian TR [thioredoxin reductase] can function as a peroxynitrite reductase." In performing the study, as disclosed in the Results section at page 265, left-hand column, Arteel infuses peroxynitrite to maintain a 0.2 pM steady-state concentration in potassium phosphate buffer. Arteel uses benzoate hydroxylation and nitrite formation as indices of oxidation reactions of peroxynitrite and of peroxynitrite reduction. Arteel particularly notes that when selenocystine or ebselen are present in the reaction mixture, there is a significant suppression of benzoate hydroxylation and an increase in nitrite formation until the NAPDH was oxidized. Arteel particularly specifies that the addition of thioredoxin did not enhance these effects (page 264, in the Abstract). Moreover, on page 265, right-hand column, Arteel discloses that. "Addition of TR to ebselen had no effect under these conditions (Y)."

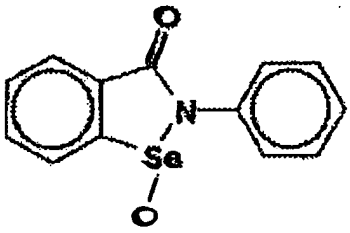
Therefore, Arteel should be considered to be nonenabling for processes wherein thioredoxin is present as Arteel does not teach or suggest any need for having the thioredoxin present in the reaction.

Appellant further notes the prior art at most teaches that ebselen is an inhibitor of thioredoxin reductase, and that ebselen selenoxide can be a substrate. However, Appellant's claimed subject matter does not include ebselen selenoxide. Arteel shows no effect of thioredoxin on reduction of ebselen selenoxide by NADPH and thioredoxin reductase.

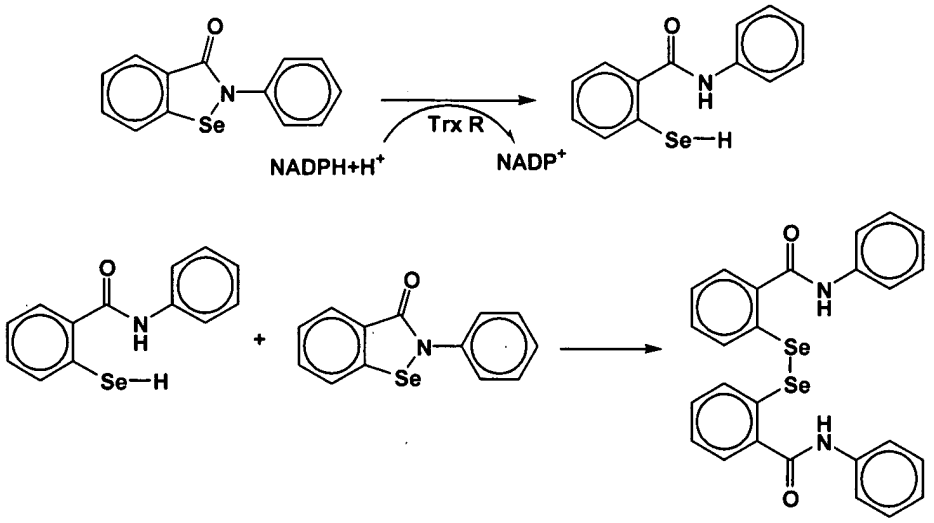
To assist a further understanding of Appellant's invention, Appellant notes that ebselen has the following formula:



Ebselen selenoxide has the following formula:



Moreover, the reaction of ebselen in Appellant's system does not form ebselen selenoxide, the compound disclosed in Arteel, but produces compounds according to the following reaction scheme, as disclosed on pages 13 and 14 of Appellant's specification.



Therefore, summarizing the above, Arteel discloses that:

(1) Ebselen inhibits thioredoxin reductase, and it is therefore expected that addition of ebselen would shut off effects of thioredoxin reductase.

(2) The strong oxidant peroxynitrite is reduced by thioredoxin reductase provided that ebselen is present. The mechanism being that ebselen is oxidized to ebselen selenoxide which is reduced by thioredoxin reductase.

(3) There is no effect by adding thioredoxin.

In contrast to the prior art utilized in the rejection, the present invention recognizes and demonstrates that ebselen is a substrate being reduced by NADPH and thioredoxin reductase with a low Km-value meaning that it is a very good substrate undergoing unlimited cycles of oxidation/reduction in the presence of hydrogen peroxide without affecting the activity of the enzyme. The reduced ebselen is called ebselen selenol and has the Se-N bond broken by reduction. The selenol is oxidized back to ebselen by hydrogen peroxide or another peroxide and a new cycle starts. The reaction is ultimately driven by NADPH. Reduced thioredoxin strongly enhances the thioredoxin reductase reaction which is also

proven by determination of the rate of reduction of ebselen by reduced thioredoxin using kinetics with tryptophan fluorescence. The result, never seen before, is that ebselen is a very efficient oxidant of reduced thioredoxin.

Thus, Appellant submits that it is by no means clear that ebselen would be a substrate for thioredoxin or thioredoxin reductase following any disclosure in Arteel, or any of the other documents utilized in the rejection. The substrate used in Arteel's work is selenocysteine, which is different from the selenocystine of Bjornstedt.

Appellant respectfully submits that one having ordinary skill in the art would not have found it obvious to have combined the disclosures to arrive at the claimed subject matter. There is no teaching or suggestion in the documents utilized in the rejection that mammalian thioredoxin reductase is a selenoenzyme and there is therefore no possibility of deducing that ebselen would be a substrate. In fact, most people testing thioredoxin reductase at the time of Appellant's invention would buy the bacterial enzyme, which was commercially available from IMCO in Stockholm as the only source and they would have seen no reactions with ebselen. Appellant's experiments utilized preparations of mammalian thioredoxin reductase from human placenta or calf thymus and with that Appellant observed that ebselen is a substrate. An extremely fast reaction with thioredoxin was part of Appellant's findings.

Muller and Schewe are utilized in the rejection for the assertion that they disclose that ebselen mimics glutathione peroxidase. While it may be true that ebselen mimics glutathione peroxidase, that is not a major concern relative to Appellant's recited methods as the issue relates to the fact that one having ordinary skill in the art at the time of Appellant' invention

would not have knowledge of ebselen being a thioredoxin reductase substrate, or a thioredoxin oxidant.

There is no supporting disclosure in the documents of record, which documents include as an author one of the coinventors of the present application, i.e., Arne Holmgren, who is a co-author of Bjornstedt and Kumar, to adequately provide a basis for the rejection.

There is no reason why one having ordinary skill in the art would arrive at a method for reducing a peroxide comprising combining thioredoxin, thioredoxin reductase, NAPDH and a substrate *in vitro* under conditions to reduce the peroxide, the substrate comprising a substance selected from the group consisting of a compound represented by the general formulas recited in claim 18 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Further, the rejection does not establish that one having ordinary skill in the art would arrive at Appellant's recited method in claim 27 which further recites that the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one (also referred to as ebselen) or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Therefore, the obviousness rejection is without appropriate basis, and should be withdrawn.

Accordingly, the obviousness rejection should be withdrawn.

(E) Arguments for Claims 19 and 28

The rejection of claims 19 and 28 under 35 U.S.C. 103(a) as being unpatentable over the combined teachings of Arteel, Bjornstedt and Kumar and in view of Muller and Schewe is in error, the decision of the Examiner to finally reject these claims should be reversed, and the application should be remanded to the Examiner.

Appellant's independent claim 19 is directed to a method of preventing peroxidation of a substance comprising combining thioredoxin, thioredoxin reductase and NADPH with a substrate *in vitro* under conditions to prevent peroxidation of the substance , the substrate being selected from the group consisting of a compound represented by the general formulas recited in claim 19 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof, and a hydrate thereof and a solvate thereof. Claim 28 further recites that the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one (also referred to as ebselen) or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

In this ground of rejection, the Examiner contends that:

> It would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made to use the process of Arteel et al, and Bjornstedt et al, wherein in vitro experiment is expressly done using NADPH, selenocystine and thioredoxin similar to claimed herein, by using ebselen, as taught by Muller et al and Schewe, because the latter references are expressly teaching that ebselen mimics glutathione peroxidase like activities, and Kumar et al is expressly teaching that selenite is a substrate for thioredoxin reductase and NADPH, and Bjornstedt et al expressly teaching that selenium is an essential element to have anti oxidant property, ebselen is having glutathione peroxidase like activity in vitro, it is obvious that ebselen when reacted with thioredoxin reductase and NADPH, would result in a method similar to claimed herein, absent evidence to the contrary.

The rejection further contends that:

> Thus, as noted in the rejection, Bjornstedt does not disclose method as recited in Applicants' claims which include the recited compounds let alone teach or suggest that compounds having a structure as ebselen would be a substrate, an inhibitor or most likely not show any reactivity.

The rejection further contends referring to Bjornstedt, ".....it is selenium which is important part of the reaction and other reference are expressly teaching that selenium is active part, and Muller is teaching ebselen to be behaving like glutathione peroxidase like activity, thus suggesting similar process as claimed herein."

Still further, referring to Kumar et al., the rejection notes that the structure is different but contends that, "it is selenium that is important and the references are expressly teaching equivalence of selenite, ebselen having glutathione peroxidase like activity."

Regarding Arteel not using ebselen, the rejection asserts that, "it is selenium which is important and inasmuch as ebselen is taught in a similar glutathione peroxidase like activity, the method claim is obvious as a whole, absent evidence to the contrary.

In contrast to the Examiner's assertions, Appellant again points out, as argued above, that the prior art shows ebselen as being an inhibitor and not a substrate. Therefore, the assertions in the rejection that it is selenium that is important and the references expressly teaching that selenium is active part is without appropriate basis. The rejection does not address that the prior art, such as Arteel and Engman specifically disclose that ebselen is an inhibitor. The contention set forth in the rejection that it is selenium that is important is like saying that any compound that contains carbon would be useful to cure a disease merely because one compound capable of curing the disease contains carbon.

Still further, Appellant submits that the rejection is not clear as to how the documents are being combined. The rejection does not indicate what is meant by the combined teachings of Arteel, Bjornstedt, and Kumar. Moreover, the rejection does not explain how the various disclosures can be combined, and how Appellant's process can be arrived at when Arteel discloses that ebselen is an inhibitor. Certainly, the rejection must address this issue without utilizing Appellant's disclosure to support the rejection.

Moreover, Appellant notes that, as disclosed in Bjornstedt at page 11761, right column, third full paragraph, Bjornstedt is directed to the investigation of whether thioredoxin reductase and thioredoxin can reduce lipid hydroperoxides and if low molecular weight selenium compounds could act as charge transfer catalysts. Bjornstedt discloses that this could be an important alternative pathway for the detoxification of hydroperoxides in addition to GSH-Px-mediated reduction. Thus, as noted in the rejection, Bjornstedt does not disclose methods as recited in Appellant's claims which include the recited compounds let alone teach or suggest that compounds having a structure such as ebselen would be a substrate, an inhibitor or most likely not show any reactivity. In fact, ebselen is not a substrate for a majority of the world's thioredoxin reductases found in all bacteria, plants, yeast, etc. These thioredoxin reductases do not reduce ebselen and, in fact, ebselen acts as an inhibitor.

Regarding Kumar, Appellant notes that Kumar relates to the fact that sodium selenite is a redox cycling agent, and does not disclose methods as recited in Appellant's claims which include the recited compounds let alone teach or suggest that compounds having a

structure such as ebselen would be a substrate, an inhibitor or most likely not show any reactivity.

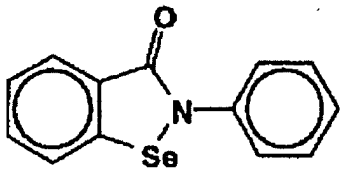
Appellant submits that Arteel does not teach any methods wherein ebselen is used as a substrate for thioredoxin reductase. Arteel performs experiments with respect to the activity of mammalian thioredoxin reductase using a peroxynitrite reductase. Thus, at page 264, at the bottom of the left-hand column, Arteel discloses, "Here we investigated whether mammalian TR [thioredoxin reductase] can function as a peroxynitrite reductase." In performing the study, as disclosed in the Results section at page 265, left-hand column, Arteel infuses peroxynitrite to maintain a 0.2 pM steady-state concentration in potassium phosphate buffer. Arteel uses benzoate hydroxylation and nitrite formation as indices of oxidation reactions of peroxynitrite and of peroxynitrite reduction. Arteel particularly notes that when selenocystine or ebselen are present in the reaction mixture, there is a significant suppression of benzoate hydroxylation and an increase in nitrite formation until the NAPDH was oxidized. Arteel particularly specifies that the addition of thioredoxin did not enhance these effects (page 264, in the Abstract). Moreover, on page 265, right-hand column, Arteel discloses that. "Addition of TR to ebselen had no effect under these conditions (Y)."

Therefore, Arteel should be considered to be nonenabling for processes wherein thioredoxin is present as Arteel does not teach or suggest any need for having the thioredoxin present in the reaction.

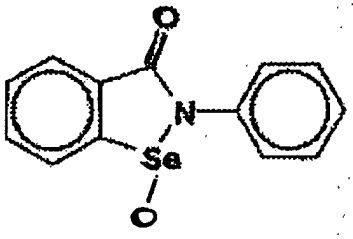
Appellant further notes the prior art at most teaches that ebselen is an inhibitor of thioredoxin reductase, and that ebselen selenoxide can be a substrate. However, Appellant's

claimed subject matter does not include ebselen selenoxide. Arteel shows no effect of thioredoxin on reduction of ebselen selenoxide by NADPH and thioredoxin reductase.

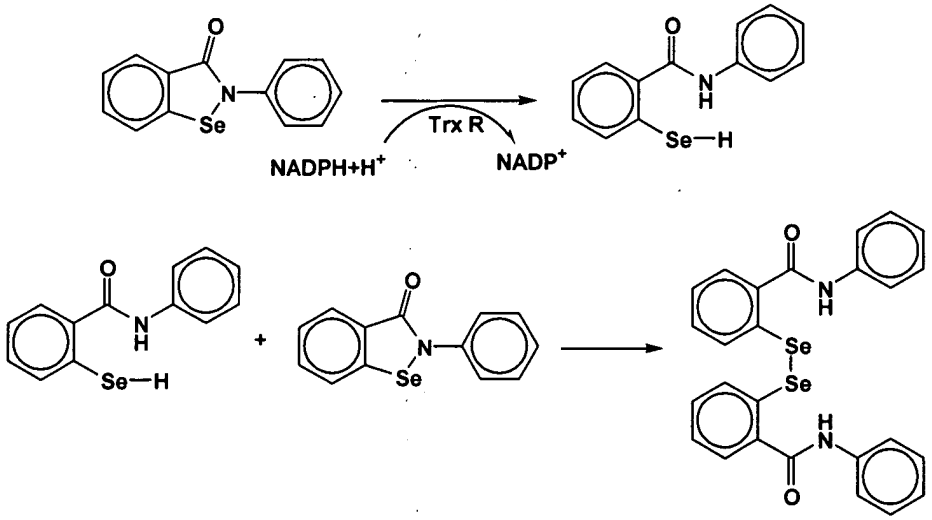
To assist a further understanding of Appellant's invention, Appellant notes that ebselen has the following formula:



Ebselen selenoxide has the following formula:



Moreover, the reaction of ebselen in Appellant's system does not form ebselen selenoxide, the compound disclosed in Arteel, but produces compounds according to the following reaction scheme, as disclosed on pages 13 and 14 of Appellant's specification.



Therefore, summarizing the above, Arteel discloses that:

(1) Ebselen inhibits thioredoxin reductase, and it is therefore expected that addition of ebselen would shut off effects of thioredoxin reductase.

(2) The strong oxidant peroxynitrite is reduced by thioredoxin reductase provided that ebselen is present. The mechanism being that ebselen is oxidized to ebselen selenoxide which is reduced by thioredoxin reductase.

(3) There is no effect by adding thioredoxin.

In contrast to the prior art utilized in the rejection, the present invention recognizes and demonstrates that ebselen is a substrate being reduced by NADPH and thioredoxin reductase with a low Km-value meaning that it is a very good substrate undergoing unlimited cycles of oxidation/reduction in the presence of hydrogen peroxide without affecting the activity of the enzyme. The reduced ebselen is called ebselen selenol and has the Se-N bond broken by reduction. The selenol is oxidized back to ebselen by hydrogen peroxide or another peroxide and a new cycle starts. The reaction is ultimately driven by NADPH. Reduced thioredoxin strongly enhances the thioredoxin reductase reaction which is also proven by determination of the rate of reduction of ebselen by reduced thioredoxin using kinetics with tryptophan fluorescence. The result, never seen before, is that ebselen is a very efficient oxidant of reduced thioredoxin.

Thus, Appellant submits that it is by no means clear that ebselen would be a substrate for thioredoxin or thioredoxin reductase following any disclosure in Arteel, or any of the other documents utilized in the rejection. The substrate used in Arteel's work is selenocysteine, which is different from the selenocystine of Bjornstedt.

Appellant respectfully submits that one having ordinary skill in the art would not have found it obvious to have combined the disclosures to arrive at the claimed subject matter. There is no teaching or suggestion in the documents utilized in the rejection that mammalian thioredoxin reductase is a selenoenzyme and there is therefore no possibility of deducing that ebselen would be a substrate. In fact, most people testing thioredoxin reductase at the time of Appellant's invention would buy the bacterial enzyme, which was commercially available from IMCO in Stockholm as the only source and they would have seen no reactions with ebselen. Appellant's experiments utilized preparations of mammalian thioredoxin reductase from human placenta or calf thymus and with that Appellant observed that ebselen is a substrate. An extremely fast reaction with thioredoxin was part of Appellant's findings.

Muller and Schewe are utilized in the rejection for the assertion that they disclose that ebselen mimics glutathione peroxidase. While it may be true that ebselen mimics glutathione peroxidase, that is not a major concern relative to Appellant's recited methods as the issue relates to the fact that one having ordinary skill in the art at the time of Appellant' invention would not have knowledge of ebselen being a thioredoxin reductase substrate, or a thioredoxin oxidant.

There is no supporting disclosure in the documents of record, which documents include as an author one of the coinventors of the present application, i.e., Arne Holmgren, who is a co-author of Bjornstedt and Kumar, to adequately provide a basis for the rejection.

There is no reason why one having ordinary skill in the art would arrive at a method of preventing peroxidation of a substance comprising combining thioredoxin, thioredoxin

reductase and NADPH with a substrate *in vitro* under conditions to prevent peroxidation of the substance , the substrate being selected from the group consisting of a compound represented by the general formulas recited in claim 19 and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof, and a hydrate thereof and a solvate thereof. Further, the rejection does not establish that one having ordinary skill in the art would arrive at Appellant's recited method in claim 29 which further recites that the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one (also referred to as ebselen) or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. Therefore, the obviousness rejection is without appropriate basis, and should be withdrawn.

Accordingly, the obviousness rejection should be withdrawn.

CONCLUSION

For the reasons set forth above, it is respectfully submitted that the Examiner has failed to establish a prima facie case of obviousness, which is a prerequisite for maintaining a rejection under 35 U.S.C. 103(a). The Board is, therefore, respectfully requested to reverse the Final Rejection, and to allow the application to issue in its present form.

Respectfully submitted,
Arne HOLMGREN et al.

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Reg. No. 33094

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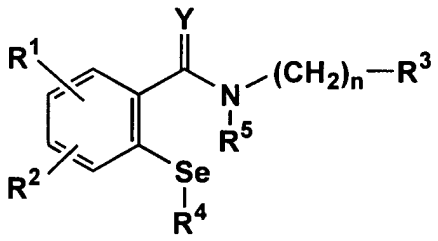
(VIII) CLAIMS APPENDIX

Claim 13. A method for reduction of a substrate with thioredoxin reductase, comprising combining the thioredoxin reductase, the substrate and NADPH *in vitro* under conditions to reduce the substrate, the substrate comprising a substance selected from the group consisting of a compound represented by the following general formula (1) or (1') and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof:



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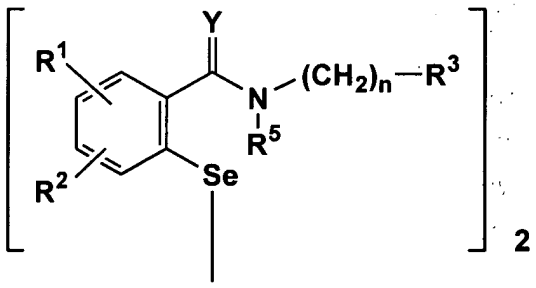
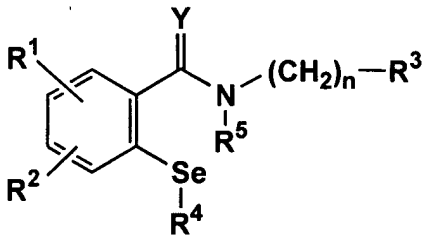


(1')

wherein R^1 and R^2 independently represent a hydrogen atom, a halogen atom, a trifluoromethyl group, a nitro group, a C_1-C_6 alkyl group, or a C_1-C_6 alkoxyl group, or R^1 and R^2 may combine together to represent methylenedioxy group; R^3 represents an aryl group, an aromatic heterocyclic group, a 5- to 7-membered cycloalkyl group, or a 5- to 7-membered cycloalkenyl group, and the aryl group, the aromatic heterocyclic group, the cycloalkyl group, and the cycloalkenyl group may be substituted with one or more substituents; R^4 represents a hydrogen atom, a hydroxyl group, a -S-glutathione group, a -S- α-amino acid group, or an aralkyl group whose aryl moiety may be substituted with one or more substituents; R^5 represents a hydrogen atom or a C_1-C_6 alkyl group, or R^4 and R^5 may combine together to represent single bond; Y represents oxygen atom or sulfur atom; n represents an integer of from 0 to 5; and the selenium atom may be oxidized.

Claim 14. The method according to claim 13 wherein the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-selenazol-3(2H)-one or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

Claim 15. A method of enhancing peroxidase activity of thioredoxin reductase, comprising combining NAPDH, thioredoxin reductase, thioredoxin and a substrate *in vitro* under conditions to enhance peroxidase activity of thioredoxin reductase, the substrate comprising a substance selected from the group consisting of a compound represented by the following general formula (1) or (1') and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof:



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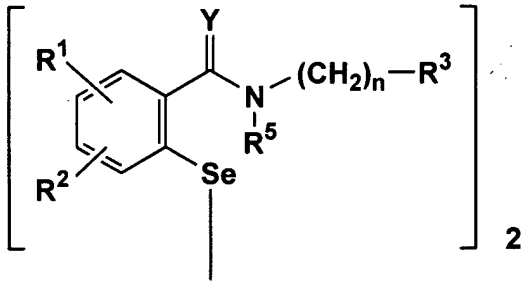
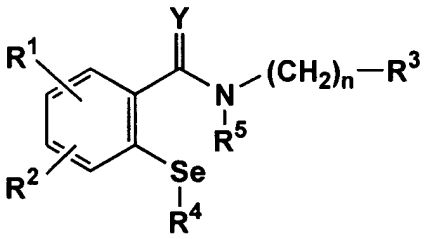


(1')

wherein R^1 and R^2 independently represent a hydrogen atom, a halogen atom, a trifluoromethyl group, a nitro group, a C_1-C_6 alkyl group, or a C_1-C_6 alkoxyl group, or R^1 and R^2 may combine together to represent methylenedioxy group; R^3 represents an aryl group, an aromatic heterocyclic group, a 5- to 7-membered cycloalkyl group, or a 5- to 7-membered cycloalkenyl group, and the aryl group, the aromatic heterocyclic group, the cycloalkyl group, and the cycloalkenyl group may be substituted with one or more substituents; R^4 represents a hydrogen atom, a hydroxyl group, a -S-glutathione group, a -S- α-amino acid group, or an aralkyl group whose aryl moiety may be substituted with one or more substituents; R^5 represents a hydrogen atom or a C_1-C_6 alkyl group, or R^4 and R^5 may combine together to represent single bond; Y represents oxygen atom or sulfur atom; n represents an integer of from 0 to 5; and the selenium atom may be oxidized.

Claim 16. The method according to claim 15 wherein the substrate comprises a substance selected from the group consisting of 2-phenyl-1,2-benzisoselenazol-3(2H)-one or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

Claim 17. A method of oxidizing reduced thioredoxin by a substrate, the method comprising combining reduced thioredoxin and a substrate *in vitro* under conditions to oxidize the reduced thioredoxin with the substrate, the substrate comprising a substance selected from the group consisting of a compound represented by the following general formula (1) or (1') and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof:



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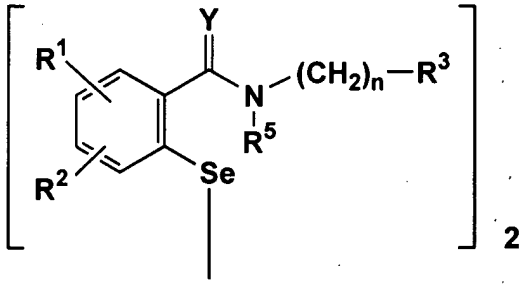
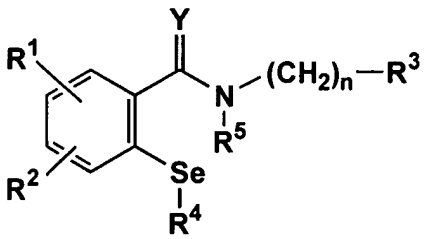


(1')

wherein R^1 and R^2 independently represent a hydrogen atom, a halogen atom, a trifluoromethyl group, a nitro group, a C_1-C_6 alkyl group, or a C_1-C_6 alkoxyl group, or R^1 and R^2 may combine together to represent methylenedioxy group; R^3 represents an aryl group, an aromatic heterocyclic

group, a 5- to 7-membered cycloalkyl group, or a 5- to 7-membered cycloalkenyl group, and the aryl group, the aromatic heterocyclic group, the cycloalkyl group, and the cycloalkenyl group may be substituted with one or more substituents; R^4 represents a hydrogen atom, a hydroxyl group, a -S-glutathione group, a -S- α-amino acid group, or an aralkyl group whose aryl moiety may be substituted with one or more substituents; R^5 represents a hydrogen atom or a C_1-C_6 alkyl group, or R^4 and R^5 may combine together to represent single bond; Y represents oxygen atom or sulfur atom; n represents an integer of from 0 to 5; and the selenium atom may be oxidized.

Claim 18. A method for reducing a peroxide comprising combining thioredoxin, thioredoxin reductase, NAPDH and a substrate *in vitro* under conditions to reduce the peroxide, the substrate comprising a substance selected from the group consisting of a compound represented by the following general formula (1) or (1') and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof:



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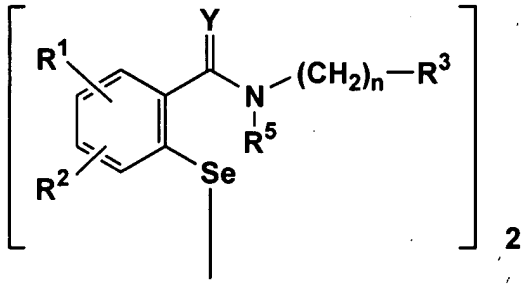
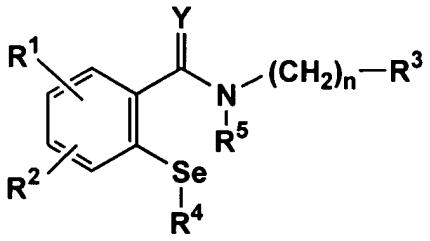


(1')

wherein R^1 and R^2 independently represent a hydrogen atom, a halogen atom, a trifluoromethyl group, a nitro group, a C_1-C_6 alkyl group, or a C_1-C_6 alkoxyl group, or R^1 and R^2 may combine together to represent methylenedioxy group; R^3 represents an aryl group, an aromatic heterocyclic group, a 5- to 7-membered cycloalkyl group, or a 5- to 7-membered cycloalkenyl group, and the aryl group, the aromatic heterocyclic group, the cycloalkyl group, and the cycloalkenyl group may be substituted with one or more substituents; R^4 represents a hydrogen atom, a hydroxyl group, a -S-glutathione group, a -S- α-amino acid group, or an aralkyl group whose aryl moiety may be substituted with one or more substituents; R^5 represents a hydrogen atom or a C_1-C_6 alkyl group, or R^4 and R^5 may combine together to represent single bond; Y represents oxygen atom or sulfur atom; n represents an integer of from 0 to 5; and the selenium atom may be oxidized.

Claim 19. A method of preventing peroxidation of a substance comprising combining thioredoxin, thioredoxin reductase and NADPH with a substrate *in vitro* under conditions to prevent peroxidation of the substance , the substrate being selected from the group consisting of a compound represented by the following general formula (1) or (1') and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof:



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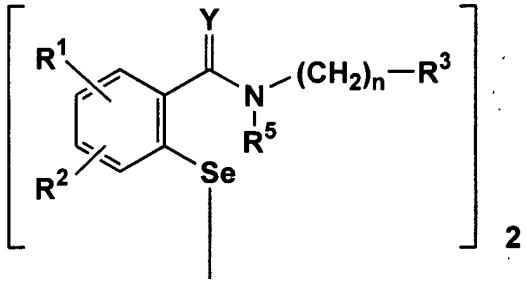

(1')

wherein R^1 and R^2 independently represent a hydrogen atom, a halogen atom, a trifluoromethyl group, a nitro group, a C_1-C_6 alkyl group, or a C_1-C_6 alkoxyl group, or R^1 and R^2 may combine together to represent methylenedioxy group; R^3 represents an aryl group, an aromatic heterocyclic group, a 5- to 7-membered cycloalkyl group, or a 5- to 7-membered cycloalkenyl group, and the aryl group, the aromatic heterocyclic group, the cycloalkyl group, and the cycloalkenyl group may be substituted with one or more substituents; R^4 represents a hydrogen atom, a hydroxyl group, a -S-glutathione group, a -S- α-amino acid group, or an aralkyl group whose aryl moiety may be substituted with one or more substituents; R^5 represents a hydrogen atom or a C_1-C_6 alkyl group, or R^4 and R^5 may combine together to represent single bond; Y represents oxygen atom or sulfur atom; n represents an integer of from 0 to 5; and the selenium atom may be oxidized.

Claim 26. The method according to claim 17 wherein the substrate comprises a substance selected from the group consisting of 2-phenyl-1 ,2-benziso-selenazol-3(2H)-one or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

Claim 27. The method according to claim 18 wherein the substrate comprises a substance selected from the group consisting of 2-phenyl-1 ,2-benziso-selenazol-3(2H)-one or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

Claim 28. The method according to claim 19 wherein the substrate comprises a substance selected from the group consisting of 2-phenyl-1 ,2-benziso-selenazol-3(2H)-one or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

(IX) Evidence Appendix

(a) U.S. Patent No. 4,418,069 to Welter et al. – entered in record in initialed Form PTO-1449 attached to Office Action mailed March 10, 2003

(b) U.S. Patent No. 4,730,053 to Dereu et al. – entered in record in initialed Form PTO-1449 attached to Office Action mailed March 10, 2003

(c) EP 0 366 990 – entered in record in initialed Form PTO-1449 attached to Office Action mailed May 31, 2006

(d) CA 02276984 – entered in record in initialed Form PTO-1449 attached to Office Action mailed May 31, 2006

(e) WO 97/26968 – entered in record in Office Action mailed May 31, 2006

(f) Arteel et al., Chem. Res. Toxicol, 1999, 12, 264-269 – entered in record in initialed Form PTO-1449 attached to Office Action mailed March 10, 2003

(g) Bjornstedt et al., Journal of Biological Chemistry, Vol. 270, No. 20, Issue of May 19, pp. 11761-11764, 1995 – entered in record in Office Action mailed May 31, 2006

(h) Kumar et al., Eur. J. Biochem, 207, 435-439, 1992 entered in recond in Office Action mailed May 31, 2006

(i) Muller et al., Biochemical Pharmacology, Vol. 33, No. 20, pp. 3235-3239, 1984 – entered in record in initialed Form PTO-1449 attached to Office Action mailed March 10, 2003

(j) Schewe, Gen. Pharmac., Vol. 26, No. 6, pp. 1153-1169, 1995 – entered in record in initialed Form PTO-1449 attached to Office Action mailed February 26, 2007

(k) Engman et al., Anticancer Res. 1997 Nov-Dec;17(6D):4599-605 (Abstract) – entered in record in initialed Form PTO-1449 attached to Office Action mailed Dcember 31, 2003

(X) Related Proceedings Appendix

Decision of the Board of Patent Appeals and Interferences, Appeal No. 2005-0936, mailed July 29, 2005, Ex parte ARNE HOLMGREN, MARJAN H. AMIRI and HIROYUKI MASAYASU

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Ex parte ARNE HOLMGREN, MARJAN H. AMIRI and HIROYUKI MASAYASU

Appeal No. 2005-0936
Application No. 09/926,218

ON BRIEF

Before ELLIS, SCHEINER and ADAMS, Administrative Patent Judges.

SCHEINER, Administrative Patent Judge.

DECISION ON APPEAL

This is an appeal under 35 U.S.C. § 134 from the final rejection of claims 13-25, the only claims remaining in the application. Claims 13-25 are reproduced in the Appendix accompanying appellants' Brief on Appeal of August 24, 2004.

The references relied on by the examiner are:

Müller et al. (Müller), "A Novel Biologically Active Seleno-Organic Compound – Glutathione Peroxidase-Like Activity *In Vitro* and Antioxidant Capacity of PZ 51 (Ebselen)," Biochemical Pharmacology, Vol. 33, No. 20, pp. 3235-3239 (1984)

Arteel et al. (Arteel), "Function of Thioredoxin Reductase as a Peroxynitrite Reductase Using Selenocystine or Ebselen," Chem. Res. Toxicol., Vol. 12, pp. 264-269 (1999).

Claims 13-25 stand rejected under 35 U.S.C. § 102 (b) as anticipated by Arteel. In addition, claims 13-25 stand rejected under 35 U.S.C. § 103 as unpatentable over Arteel and Müller.

We reverse these rejections, and raise an additional issue for consideration.

BACKGROUND

The thioredoxin/thioredoxin reductase system regulates reversible reduction-oxidation of thiol groups and maintains a constant thiol level in vivo to prevent functional depression of thiol proteins by formation of disulfide bonds and advanced peroxidation. Thioredoxin reductase reductively cleaves a disulfide bond on a target protein in the presence of NADPH and thioredoxin. Thioredoxin is a protein containing two thiol groups, and also functions as a proton donor in reduction of ribonucleotide by ribonucleotide reductase. Specification, page 1.

According to appellants, selenium compounds such as 2-phenyl-1,2-benzisoselenazol-3(2H)-one (hereinafter, ebselen) "can function as [substrates] of thioredoxin reductase by repeated self reduction-oxidation similarly to thioredoxin in the thioredoxin/thioredoxin reductase system," and can "enhance peroxidase activity of thioredoxin reductase in the presence of thioredoxin reductase and thioredoxin" (id., pages 1-2). Appellants further explain that selenium compounds were known to reduce peroxides "by [a] glutathione-like activity . . . [but], the reduction of a peroxide by glutathione peroxidase is based on [a] totally different mechanism . . . [than that of] thioredoxin reductase" (id., page 2).

DISCUSSION

The present invention is directed to a method of reducing a substrate with thioredoxin reductase (claims 13 and 14); a method of enhancing the peroxidase activity of thioredoxin reductase in the presence of NADPH, thioredoxin and a substrate (claims 15, 16, 20 and 21); a method of oxidzing reduced thioredoxin by a substrate (claim 17); a method for reducing a peroxide by combining thioredoxin, thioredoxin reductase, NADPH and a substrate (claims 18, 22 and 23); and finally, a method of

preventing peroxidation of a substance by combining the substance with thioredoxin, thioredoxin reductase, NADPH and a substrate (claims 19, 24 and 25). Claims 20-25 require that these reactions occur in vivo. The substrate in all of these claims is a selenium compound, and for purposes of this appeal, we will focus on a particular selenium compound, ebselen. The common thread that runs through all of these claims is the cyclical reduction and re-oxidation of the substrate (in this case, ebselen) in the presence of thioredoxin reductase and NADPH; i.e., ebselen is repeatedly reduced to ebselen selenol, and re-oxidized to ebselen.

The examiner rejected all of the claims under 35 U.S.C. § 102 (b) as anticipated by Arteel. In view of its brevity, we reproduce the examiner's rejection in its entirety (Answer, pages 3-4):

> Arteel is teaching a substrate for thioredoxin reductase which has the same formula as claimed herein, see the title, wherein Ebselen is mentioned. This is the same as the 2-phenyl-1,2-benzisoselen[a]zol-3(2H). See line 1 of the abstract, wherein mammal is cited, column 1, 2nd paragraph, lines 1-3 and 7-8, column 2, 1st paragraph on page 264, teach all the elements of the instant claims.

Arteel describes the activity of mammalian thioredoxin reductase as a peroxynitrite reductase. Appellants concede that Arteel performs experiments with thioredoxin reductase and ebselen, but argue essentially that under the conditions used in the reference, i.e., in the presence of both ebselen and peroxynitrite, "[t]he strong oxidant peroxynitrite is reduced by thioredoxin reductase . . . and ebselen is oxidized to ebselen selenoxide which is reduced by thiodoxin reductase" (Brief, page 14). Appellants emphasize that the substrates of the present claims "do not include ebselen selenoxide" (id., page 13).

In contrast to Arteel's system, according to appellants, "the reaction of ebselen in [a]ppellant's system does not form ebselen selnoxide" (id.); instead, "ebselen is a substrate being reduced by NADPH and thioredoxin reductase . . . [and] undergo[es] unlimited cycles of oxidation/reduction in the presence of hydrogen peroxide without affecting the activity of the enzyme. The reduced ebselen is called ebselen selenol and . . . is oxidized back to ebselen by hydrogen peroxide . . . and a new cycle starts . . . driven by NADPH" (id., page 16).

Finally, appellants point out that none of Arteel's reactions occurs in vivo, as required by claims 20-25.

The examiner's response to these arguments is to assert, for the first time, and without further explanation, that Arteel's "Figure 6[ ] is the same method as claimed by [ ] appellants" (Answer, page 6); and that page 267, column 1, last paragraph, . . . expressly [teaches] that ebselen is [a] substrate" (id.). On cursory inspection, these excerpts of Arteel appear to concern reduction of a diselenide (ebselen is not a diselenide) on the one hand, and reduction of ebselen selenoxide (with the reaction cycling between ebselen and ebselen selenoxide, rather than cycling between ebselen and ebselen selenol, as required by the present claims) on the other.

With respect claims 20-25, directed to in vivo methods, we agree with appellants that the mere description of thioredoxin reductase as a mammalian enzyme is not a description of an in vivo reaction involving ebselen.

"[E]very limitation of a claim must identically appear in a single prior art reference for it to anticipate the claim." Gechter v. Davidson, 116 F.3d 1454, 1457, 43 USPQ2d 1030, 1032 (Fed. Cir. 1997). Moreover, "the Patent Office has the initial burden of

coming forward with some sort of evidence tending to disprove novelty." In re Wilder, 429 F.2d 447, 450, 166 USPQ2d 545, 548 (CCPA 1970). We find that the examiner's initial burden of establishing a prima facie case of anticipation has not been met. The rejection of claims 13-25 under 35 U.S.C. § 102 (b) as anticipated by Arteel is reversed.

Obviousness

The examiner also rejected claims 13-25 under 35 U.S.C. § 103 as unpatentable over the combined teachings of Arteel and Müller. According to the examiner, Arteel does not teach that ebselen "is also an enhancer of the peroxidase activity of thioredoxin reductase" (Answer, page 4), and cites Müller as teaching that ebselen is known to be an enhancer of peroxidase activity.

Nevertheless, Müller concerns the glutathione peroxidase-like activity of ebselen, and the examiner has not begun to explain how this would be relevant to the claimed invention, especially in light of the present specification's teaching that "the reduction of a peroxide by glutathione peroxidase is based on [a] totally different mechanism . . . [than that of] thioredoxin reductase" (Specification, page 2). The examiner's reliance on Müller does nothing to resolve the underlying deficiencies of Arteel's disclosure.

The initial burden of presenting a prima facie case of obviousness rests on the examiner. In re Oetiker, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992). The rejection under 35 U.S.C. § 103 is reversed because the examiner has not established that the subject matter of claims 13-25 would have been suggested by the prior art.

AN ADDITIONAL ISSUE

It appears from the record that ebselen has been known in the art for some time as an anti-oxidant, and may have been administered in vivo prior to the effective filing date of the present application. It is not clear from the record whether appellants and the examiner have determined whether or not this is the case, and if so, whether administration of ebselen in vivo inherently results in the activities required by the claims.

CONCLUSION

On consideration of the record, the rejections of the claims under 35 U.S.C. §§ 102 (b) and 103 are reversed.

REVERSED

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A2

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(54) **Stabile parenteral solution of 2-phenyl-1.2-benzisoselenazol-3(2H)-one and process for producing the same.**

(57) The present invention is related to new parenteral preparations of 2-phenyl-1.2-benzisoselenazol- 3(2H)-one (Ebselen) comprising additionally one orseveral phospholipids and, possibly, one or several auxiliary agents. The invention is further related to a process for producing such solutions and their use in the preparation of drug preparations ob Ebselen.

EP 0 366 990 A2

Stabile parenteral solution of 2-phenyl-1.2-benzisoselenazol-3(H)-one and process for producing the same

The present invention is related to new solutions which may be parenterally administer and which comprise 2-phenyl 1.2-benzisoselenazol-3(2H)-one (Ebselen) in combination with one or several phospholipids in a weight proportion of from 1:2500 to 1:15 and, possibly, one or several auxiliary agents. The invention is further related to the production of such solutions as well as their use in the production of drug preparations comprising 2-phenyl-1.2-benzisoselenazol-3(2H)-one (Ebselen) and one or several phospholipids.

Ebselen is a known product (DE-PS 3027073). It may be produced by the process of R. Weber and M. Renson, Bulletin de la Soc. Chim. de France 1976 (7/8), pgs. 1124-1126, by subjecting 2-methylseleno-N-phenyl-benzamide to reaction with phosphorous pentachloride and subsequently hydrolysing the obtained product. Preparations comprising Ebselen may be used in the treatment of numerous diseases such as the prophylaxis and therapy of infection diseases, the therapy of malignent tumors (DE-0S 3638124), for stimulating the immuno system or for the treatment of selen deficiency diseases. Further attention is drawn to the application of the anti-artheriosclerotic and anti-inflammatory properties of Ebselen and their application in the therapy of rheumatic diseases (DE-0S 3027073). Ebselen ist furthermore an important agent useful in the therapy of deficiencies caused by oxydative stress (DE-0S 3616920) such is lever deficiencies, cardiac infarction, psoriasis and diseases caused by radiation. There is known also a drug preparation for the topical use of Ebselen (DE-0S 3620674), which may be used in the external treatment of inflammatory and allergic skin diseases such as psoriasis.

The broad spectrum of properties is in contrast to a very low solubility of Ebselen in water. Due thereto the use of Ebselen in the form of parenteral solutions is prevented. Preparations comprising organic solvents containing Ebselen dissolved therein do not provide satisfactory results because diluting such solutions with water for injections or with physiological saline solutions cause precipitation of crystals of Ebselen.

It has now been found that surprisingly stabile aqueous solutions of 2-phenyl-1.2-benzisoselenazol-3-(2H)-one (Ebselen) having a physiological pH may be produced by combining Ebselen with one or several natural or synthetic phospholipids and this in a weight proportion of Ebselen to phospholipid amounting to a ratio ranging from 1:2500 to 1:15. Possibly, further auxiliary agents may be added.

In this way, new aqueous solutions of Ebselen in combination with one or several phospholipids are formed. Such solutions are very suitable for parenteral administration (for instance for intramuscular or intravenous administration) and such solutions show a long existing effectiveness.

For producing such solutions, the components thereof are added to each other and stirred to produce homogenous solutions in usual manners, for instance by aid of high pressure homogenators. In some instances it is possible to obtain the solutions by simple stirring. Another possibility to produce the solutions is treatment with ultrasonics or by using the so called "French Press".

Products to produce isotonic solutions may be added before or after the preparation of the homogenous solutions. Such products are sodium chloride, glucose or the like. It may be advantageous to add a base, for instance soda lye or a buffer agent in order to produce a pH close to the physiological pH. The solutions thus prepared may be sterilized in usual manners and filled into ampoules as usual.

In view of the sensitivity of the phospholipids against light and oxygen, it may be preferable to work with the exclusion of oxygen and in a protective atmosphere, and with the exclusion of light.

Both natural and synthetic phospholipids may be used. Natural phospholipids (of plant or animal origin) are in particular phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inosit, phosphatidyl glycol, cardiolipine or plasmalogenes, which products may be recovered from soybeans or from eggs. Further useful are mixtures of several such phospholipids such as the trade products
Phospholipon (R) 100 (95% natural phosphatidylcholine from soybeans)
Phospholipon (R) 100 H (98% fully hydrogenated phosphatidylcholine from soybeans) Phospholipon (R) 80 (phospholipids from soybeans comprising 76% of phosphatidylcholine and 12% of phosphatidylethanolamine).

Synthetic phosphatides are for instance:
dihexadecanoylphosphatidylcholine,
ditetradecanoylphosphatidylcholine,
dioleylphosphatidylcholine,
dilinolylphosphatidylcholine,
in particular

dipalmitoylphosphatidylcholine and
dipalmitoylphosphatidylglycerol.

Auxiliary agents are for instance cholesterol, derivatives of bile acids and salts thereof, benzylalcohol, neutral oils (Miglyol 812) and glycerol.

The production of the preparations according to the present invention is further illustrated in the following examples.

Example 1	
Ebselen	0.110 g
DPPC (dipalmitoylphosphatidylcholine)	13.330 g
DPPG (dipalmitoylphosphatidylglycerol)	1.330 g
cholesterol	6.450 g
buffering agent to pH4	up to 1000 ml

Ebselen, DPPC, DPPG and cholesterol are dissolved in a mixture of 1 part of methanol and 1 part of chloroform. The solvent is removed and the resulting film is hydrated with buffer under an inert gas. Glas balls are added and liposomes are formed with stirring. They are filtered in usual manner under sterile conditions and filled into ampoules.

Example 2	
Ebselen	0.150 g
DPPC	18.180 g
DPPG	1.818 g
cholesterol	8.790 g
water for injections	up to 1000 ml

The products are mixed and further processed as described in example 1.

Example 3	
Ebselen	0.250 g
DPPC	30.300 g
DPPG	3.030 g
cholesterol	14.650 g
buffering agent	up to 1000 ml

The products are mixed and further processed as described in example 1.

Example 4	
Ebselen	0.330 g
DPPC	39.970 g
DPPG	3.997 g
cholesterol	19.338 g
buffering agent	up to 1000 ml

The products are mixed and further processed as described in example 1.

Example 5	
Ebselen	0.400 g
DPPC	48.480 g
DPPG	4.848 g
cholesterol	23.440 g
buffering agent	up to 1000 ml

The products are mixed and further processed as described in in example 1.

Example 6	
Ebselen	0.430 g
DPPC	50.170 g
DPPG	5.017 g
cholesterol	24.112 g
buffering agent	up to 1000 ml

The products are mixed and further processed as described in in example 1.

Example 7	
Ebselen	0.100 g
Phospholipon (R) 100	45.215 g
sodiumdesoxycholate	17.621 g
benzylalcohol	15.700 g
water for injections	up to 1000 ml

Ebselen and Phospholipon 100 are dissolved in ethanol. After removal of the solvent under vacuum, the resulting mixture is stirred into a solution of sodiumdesoxycholate. After the addition of benzylalcohol and water as described in example 1, the solution is filtered under sterile conditions and filled into ampoules.

Example 8	
Ebselen	0.300 g
Phospholipon (R) 100	116.900 g
sodiumdesoxycholate	45.900 g
benzylalcohol	15.700 g
water for injections	up to 1000 ml

The products are mixed and further processed as described in in example 7.

Example 9	
Ebselen	0.300 g
Phospholipon (R) 100	116.900 g
glycocholin acid	58.200 g
NaOH	5.000 g
benzylalcohol	15.700 g
water for injections	up to 1000 ml

Example 10	
Ebselen	0.300 g
Phospholipon (R) 80	116.900 g
imrocholin acid	64.370 g
NaOH	5.000 g
benzylalcohol	15.700 g
water for injections	up to 1000 ml

The products are mixed and further processed as described in in example 7.

Example 11	
Ebselen	0.450 g
Phospholipon (R) 100	110.440 g
sodium cholate	40.125 g
benzylalcohol	15.700 g
water for injections	up to 1000 ml

The products are mixed and further processed as described in in example 7.

Example 12	
Ebselen	0.500 g
Phospholipon (R) 80	108.700 g
water for injections	up to 1000 ml

Ebselen and Phospholipon (R) 100 are dispersed with stirring in water for injection purposes. The resulting mixture is treated in the high pressure homogenator. The further subsequent filtration under sterile conditions and filling into ampoules is executed as described in example 1.

Example 13	
Ebselen	0.420 g
Phospholipon (R) 80	111.250 g
water for injections	up to 1000 ml

The products are mixed and further processed as described in in example 12.

Example 14	
Ebselen	0.200 g
lecithine	20.000 g
Miglyol 812	170.000 g
glycerol	16.000 g
water for injections	up to 1000 ml

Ebselen is dissolved in Miglyol 812 and lecithine (solution I). Glycerol is added to the water for injection purposes (solution II). Both solutions are mixed and treated in the high pressure homogenator. The resulting emulsion is stirilised in the autoclave and filled into ampoules as usual.

Example 15	
Ebselen	0.500 g
lecithine	24.000 g
Miglyol 812	200.000 g
glycerol	32.000 g
water for injections	up to 1000 ml

The products are mixed and further processed as described in in example 14.

Example 16	
Ebselen	1.000 g
lecithine	24.000 g
Miglyol 812	200.000 g
glycerol	32.000 g
water for injections	up to 1000 ml

The products are mixed and further processed as described in in example 14.

Example 17	
Ebselen	2.000 g
lecithine	32.000 g
Miglyol 812	220.000 g
glycerol	37.000 g
water for injections	up to 1000 ml

The products are mixed and further processed as described in in example 14.

Claims

1. Stabile parenteral solutions of 2-phenyl-1.2-benzisoselenazol-3(2H)-one, characterized in that they contain a water-soluble combination of 2-phenyl-1.2-benzisoselenazol-3(2H)-one with one or several phospholipids.

2. Solution according to claim 1, characterized in that the weight proportion of 2-phenyl-1.2-benzisoselenazol-3(2H)-one and the phospholipid or phospholipids in the solution is between 1:2500 to 1:15.

3. Solution according to claim 1 or 2, characterized in that the phospholipid or the phospholipids represent natural or synthetic phospholipids.

4. Solution according to claims 1 to 3, characterized in that it comprises as natural phospholipid the compound soybean lecithine or egg lecithine or a highly purified fraction thereof.

5. Solution according to claims 1 to 3, charcterized in that the synthetic phospholipid is phosphatidyl choline, phosphatidyl glycerol or a mixture thereof.

6. Process for producing drug preparations as claimed in claims 1 to 5, characterized in that 2-phenyl-1.2-benzisoselenazol-3(2H)-one (Ebselen) is mixed with the phospholipid or a mixture of several phospholipids and, possibly, one or several additional auxiliary agents in known manner, the mixture is added to a physiological saline solution, the resulting physiological solution is rendered neutral, possibly by means of additional auxiliary agents, and the resuling solution is rendered sterile.

7. The use of solutions according to any of claims 1 to 5 comprising a combination of 2-phenyl-1.2-benzisoselenazol-3(2H)-one (Ebselen) and one or several phospholipids and, possibly, one or several auxiliary agents, for the preparation of drugs.

(19) Canadian Intellectual Property Office
An Agency of Industry Canada

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Un organisme d'Industrie Canada

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(43) 01.01.2000

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(22) 30.06.1999

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(30) H10-186234 JP 01.07.1998

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(54) INHIBITEUR DE CYCLO-OXYGENASE
(54) CYCLOOXYGENASE INHIBITOR

(57)

A cyclooxygenase-2-inhibitor containing as an active ingredient a compound having-cyclooxygenase-2-inhibitory activity and exhibiting low toxicity that causes only minor side effects. The active compound is represented by the following formula (1) or (1'): <IMG> wherein R1 represents a hydrogen atom or a C1-C3 alkyl group; R2 represents a hydrogen atom, a hydroxyl group, an organic group capable of being bound through its thiol group within the molecule, or R1 and R2 may loin to each other to form a single bond; R3 represents a hydrogen atom, a halogen atom, a C1-C3 alkyl group, a C1-C3 alkoxyl group, a trifluoromethyl group, or a nitro group; each of R4 and R5, which may be identical to or different from each other, represents a hydrogen atom, a halogen atom, a C1-C4 alkoxyl group, a trifluoromethyl group, or R4 and R5 may loin to each other to form a methylenedioxy group

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CANADIAN INTELLECTUAL PROPERTY OFFICE

(12) (19) (CA) **Demande-Application**

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(43) 2000/01/01

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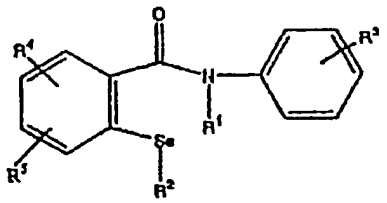
(51) Int.Cl.[6] A61K 31/41, A61K 38/38, A61K 38/17, A61K 45/06, A61K 38/06, A61K 38/04, A61K 38/00
(30) 1998/07/01 (H10-186234) JP

(54) **INHIBITEUR DE CYCLO-OXYGENASE**
(54) **CYCLOOXYGENASE INHIBITOR**



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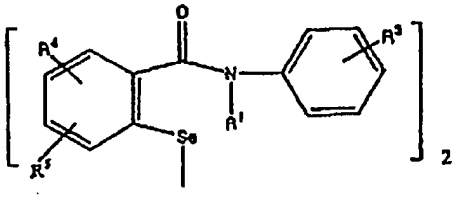
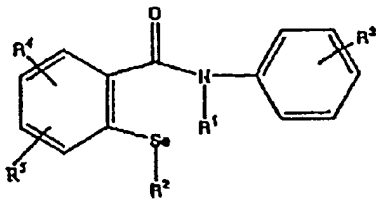


(1')

(57) A cyclooxygenase-2-inhibitor containing as an active ingredient a compound having-cyclooxygenase-2-inhibitory activity and exhibiting low toxicity that causes only minor side effects. The active compound is represented by the following formula (1) or (1'): (see formulae 1 and 1') wherein R^1 represents a hydrogen atom or a C1-C3 alkyl group; R^2 represents a hydrogen atom, a hydroxyl group, an organic group capable of being bound through its thiol group within the molecule, or R^1 and R^2 may loin to each other to form a single bond; R^3 represents a hydrogen atom, a halogen atom, a C1-C3 alkyl group, a C1-C3 alkoxyl group, a trifluoromethyl group, or a nitro group; each of R^4 and R^5, which may be identical to or different from each other, represents a hydrogen atom, a halogen atom, a C1-C4 alkoxyl group, a trifluoromethyl group, or R^4 and R^5 may join to each other to form a methylenedioxy group

Abstract of the Disclosure

A cyclooxygenase-2-inhibitor containing as an active ingredient a compound having cyclooxygenase-2-inhibitory activity and exhibiting low toxicity that causes only minor side effects. The active compound is represented by the following formula (1) or (1'):



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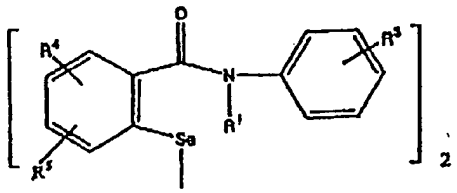


(1')

wherein R^1 represents a hydrogen atom or a C1-C3 alkyl group; R^2 represents a hydrogen atom, a hydroxyl group, an organic group capable of being bound through its thiol group within the molecule, or R^1 and R^2 may join to each other to form a single bond; R^3 represents a hydrogen atom, a halogen atom, a C1-C3 alkyl group, a C1-C3 alkoxyl group, a trifluoromethyl group, or a nitro group; each of R^4 and R^5, which may be identical to or different from each other, represents a hydrogen atom, a halogen atom, a C1-C4 alkoxyl group, a trifluoromethyl group, or R^4 and R^5 may join to each other to form a methylenedioxy group.

CYCLOOXYGENASE INHIBITOR

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a cyclooxygenase-2 inhibitor. The inhibitor inhibits synthesis of prostaglandin H and I, or synthesis of their subsequent metabolites thromboxanes, and can be used as a drug for both treatment and prevention of ischemic diseases, senile dementia, cancer, asthma, arterial sclerosis, and various inflammation diseases.

Background Art

Cyclooxygenase (prostaglandin-endoperoxide synthase) is an enzyme that catalyzes *in vivo* synthesis of prostaglandin H2 from its substrate arachidonic acid. Prostaglandin H2 is physiologically very active. From prostaglandin H2, there are produced metabolites of prostaglandin D2, E2, and F2 and metabolites of thromboxane A2 and B2, all of which are also physiologically very active.

Consequently, inhibition of the cyclooxygenase activity results in inhibition of synthesis of these metabolites. Thus, the prostaglandin H2 inhibitors can inhibit not only prostaglandin H2, but also other physiologically very active compounds, such as prostaglandin D2, E2, and F2 and thromboxane A2 and B2.

Cyclooxygenase is widely known to be involved in inflammation. For treatment of such inflammation, various cyclooxygenase inhibitors have been widely used, including

aspirin and indomethacin. However, at the time when these drugs were discovered, there was known only one type of cyclooxygenase that is ubiquitously present in the living body.

Another type of cyclooxygenase; namely, the inducible type of cyclooxygenase, has recently been discovered. This inducible type of cyclooxygenase is induced upon various stimuli and is called cyclooxygenase 2, whereas the ubiquitous type is now called cyclooxygenase 1. Furthermore, it has recently become clear that cyclooxygenase 2 is deeply involved in ischemic diseases, senile dementia, cancer, asthma, arterial sclerosis, and a variety of inflammation diseases. From these observations, cyclooxygenase-2-inhibitors are considered to be potentially very effective drugs for treatment of these diseases (G. Cirino. Biochem. Pharmacol. 55: 105-111, 1998).

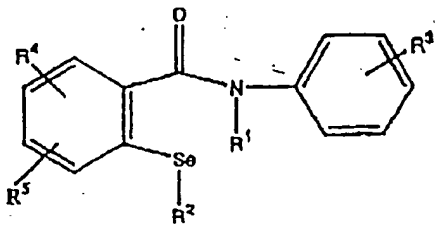
SUMMARY OF THE INVENTION

In view of the foregoing, the present invention is to provide a pharmaceutical containing, as an active ingredient thereof, a compound which inhibits cyclooxygenase 2 to thereby serve as an excellent therapeutic drug for the above-mentioned diseases, and which has low toxicity that causes only minor side effects to the extent that they are suitable for clinical use.

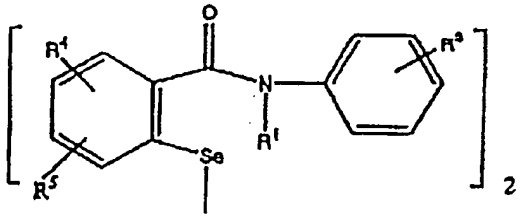
Accordingly, the present invention provides the following.

1) A cyclooxygenase-2-inhibitor comprising, as an

active ingredient, a compound represented by the following formula (1) or (1'):



(1)

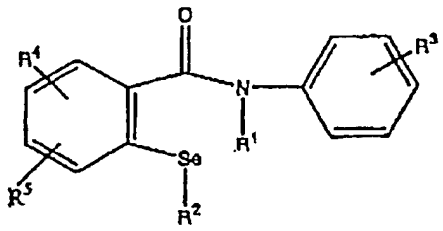


(1')

wherein R^1 represents a hydrogen atom or an alkyl group having 1-3 carbon atoms; R^2 represents a peptide or protein capable of binding through its own thiol group within the molecule, or R^1 and R^2 may join to each other to form a single bond; and R^3 represents a hydrogen atom, a halogen atom, an alkyl group having 1-3 carbon atoms, an alkoxyl group having 1-3 carbon atoms, a trifluoromethyl group, or a nitro group; and each of R^4 and R^5, which may be identical to or different from each other, represents a hydrogen atom, a halogen atom, an alkoxyl group having 1-4 carbon atoms, a trifluoromethyl group, or R^4 and R^5 may join to each other to form a methylenedioxy group; a salt thereof, or a hydrate thereof.

2) A cyclooxygenase-2-inhibitor containing, as an active ingredient, 2-phenyl-1,2-benzisoselenazol-3(2H)-one (hereinafter referred to as compound A), a salt thereof, or a hydrate thereof.

3) A cyclooxygenase-2-inhibitor comprising, as an active ingredient, a compound represented by the following formula (2):



(2)

wherein R^2 represents a peptide or protein capable of binding through its own thiol group within the molecule, and R^1, R^3, R^4, and R^5 are the same as defined above; a salt thereof; or a hydrate thereof.

4) A cyclooxygenase-2-inhibitor containing, as an active ingredient, S-(2-phenylcarbamoyl-phenylselenyl)-albumin (hereinafter referred to as compound B), a salt thereof, or a hydrate thereof.

The present invention is further directed to a method for producing a pharmaceutical composition for the therapy and/or prophylaxis of such diseases which are caused in a disturbance and/or in an influence of the cyclooxygenase-2-inhibition wherein for producing of said composition a cyclooxygenase-2-inhibitor is used as active ingredient, wherein said cyclooxygenase-2-inhibitor is characterized by the before described general formulas.

Moreover the present invention is preferably directed to a

method for producing a pharmaceutical composition for the therapy and/or prophylaxis of ischemic diseases, senile dementia, cancer, asthma, arterial sclerosis, and/or inflammation diseases wherein for producing of said composition a cyclooxygenase-2-inhibitor of the afore mentioned type is used as active ingredient.

In the frame of the present invention the term active ingredient is not only used for a single active ingredient but also for a mixture of active ingredients, which are used for the producing of the inventive composition accordingly.

Furthermore the present invention is directed to a method for therapy and/or prophylaxis of such diseases which are caused in a disturbance and/or in an influence of the cyclooxygenase-2-inhibition, wherein a pharmaceutical composition comprising a cyclooxygenase-2-inhibitor according to the afore described type as active ingredient is orally administered in a daily dosage of 100 to 2000 mg, relative to the active ingredient, for an adult.

Perferably a composition is orally administered to an adult having such a concentration of said active ingredient that a daily dosage of 200 to 1000 mg is given.

If the inventive method is used for therapy and/or prophylaxis

of such diseases which are caused in a disturbance and/or in an influence of the cyclooxygenase-2-inhibition a pharmaceutical composition comprising a cyclooxygenase-2-inhibitor as active ingredient is perorally administered in a daily dosage of 0.05 to 1000 mg, relative to the active ingredient, for an adult.

Preferably the inventive method is used for therapy and/or prophylaxis of ischemic diseases, senile dementia, cancer, asthma, arterial sclerosis, and/or inflammation diseases.

Depending on the concentration of the active ingredient which is to administer the inventive composition is given one time to three times each day.

DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention will next be described in detail.

As mentioned above, the present invention provides a cyclooxygenase-2-inhibitor containing, as an active ingredient, a compound of formula (1). Substituents for the formula (1) compound are as follows:

R^1 is a hydrogen atom or an alkyl group having 1-3 carbon atoms, with hydrogen being preferred.

R^2 is a peptide or protein capable of binding through its own thiol group within the molecule, or R^1 and R^2 may join to each other to form a single bond.

R^3 is a hydrogen atom, a halogen atom, an alkyl group having 1-3 carbon atoms, an alkoxyl group having 1-3 carbon atoms, a trifluoromethyl group, or a nitro group. Among these, hydrogen is preferred.

The present invention also provides a cyclooxygenase-2-inhibitor containing, as an active ingredient, a compound of formula (2). Substituents for the formula (2) compound are as follows:

R^2 in the above formula (2) denotes a peptide or protein capable of binding through its own thiol group within the molecule; with regard to the protein or the peptide, any protein or peptide can be used as far as it is physiologically acceptable; however, proteins present in serum such as albumins and globulins are preferred; further, among these serum proteins, the albumins are much preferred; and among the albumins the human albumin is most preferred.

The synthesis method of compound A used in the present invention has already been disclosed in Japanese Patent Publication (*kokoku*) No. 2-38591 (i.e., Japanese Patent Application Laid-open (*kokai*) No. 57-67568). The synthesis method for compound B has also been disclosed in Japanese Patent Application Laid-open (*kokai*) No. 7-233056.

Through known formulation techniques, the compound A of the present invention can be formulated in the form of a tablet, capsule, powder, granule, syrup, or preparation for injection, along with additives such as an excipient, binder, disintegrator, and solubilizer.

Specific formulation examples will be described below.

In the case of the tablet, the following formula may be used:

Tablet

compound A	50 mg
carboxymethylcellulose	25 mg
starch	5 mg
crystalline cellulose	40 mg
magnesium stearate	2 mg
total	122 mg

Compound A, when delivered by typical oral administration or parenteral administration such as injection, manifests respective expected primary pharmacological effects. In the case of oral administration, the dosage of chemical compound A is 100-2000 mg/day, preferably 200-1000 mg/day, for an adult. The dosage can be changed, depending on the severity of the patient's symptoms.

The 2-phenylcarbamoyl-phenylselenyl derivatives of the present invention and physiologically acceptable salts thereof are administered orally or parenterally. In the case of peroral administration to adults, the dosage is usually 0.05-1000 mg/day.

Toxicity

Concerning the toxicity of compounds A and B, their LD50 values were determined through use of mice and rats.

The LD50 of each compound for the mouse was not less than 6810 (mg/kg) when administered orally, and was 740 (mg/kg) when administered intraperitoneally. In the case of the rat, to reach LD50, large doses were required. These results indicate that safety levels for administration of those compounds are very high. One of the compounds, S-(2-phenylcarbamoyl-phenylselenyl)albumin, was tested for the acute toxicity. The compound was dissolved in physiological saline and was intravenously administered to mice (5 g/kg). Its LD50 was much higher than 1 g/kg, thus confirming its high safety level.

Acute toxicity

Four Wistar strain male rats at 8 weeks of age were subjected to an acute toxicity test. S-(2- phenylcarbamoyl-phenylselenyl)albumin dissolved in physiological saline was intravenously administered (1 g/kg/3 ml), and then the rats were observed for the next twenty-four hours. Within the observation period, no particular side effect was noticed, and all rats survived healthily. Furthermore, upon much higher doses of administration to both the mouse and rat, no problematic side effect was observed.

Among the compounds, S-(2-phenylcarbamoyl-phenylselenyl)albumin is expected to achieve the most promising efficacy. Prostaglandin H2 synthesized with cyclooxygenase 2 and its metabolites, such as prostaglandin D2, E2, and F2 and thromboxane A2 and B2, are placed in the arachidonate cascade, downstream of arachidonic acid. As

mentioned above, these are involved in ischemic diseases, senile dementia, cancer, asthma, arterial sclerosis, and a variety of inflammation diseases. These diseases can be treated with the 2-phenylcarbamoyl-phenylselenyl derivatives as well as with physiologically acceptable salts thereof, and excellent prognosis is expected upon their administration.

In the present invention, the 2-phenylcarbamoyl-phenylselenyl derivatives encompass compound A and compound B.

Through known formulation techniques, the 2-phenylcarbamoyl-phenylselenyl derivatives of the present invention and physiologically acceptable salts thereof can be formulated in the form of a tablet, capsule, powder, granule, syrup, or preparation for injection, along with additives such as an excipient, binder, disintegrator, and solubilizer.

The 2-phenyl-1,2-benzisoselenazol-3(2H)-one derivatives and 2-phenylcarbamoyl-phenylselenyl derivatives, both being compounds of the present invention, were tested *in vitro* for their inhibitory effects on cyclooxygenase 2 activity, which catalyzes synthesis of prostaglandin H2 from arachidonic acid. The results show remarkably strong inhibitory effects, which were much stronger than that of indomethacin (see Table 1).

Thus, the 2-phenyl-1,2-benzisoselenazol-3(2H)-one derivatives and 2-phenylcarbamoyl-phenylselenyl derivatives, both being related to the present invention, are most promising candidates as drugs for treatment of the above diseases that involve synthesis of prostaglandin H2 with

cyclooxygenase 2.

The present invention will be described next in detail by way of experimental examples, which should not be construed as limiting the scope of the present invention.

Experimental example 1

Arachidonic acid was used as the substrate in the present example. Ten μl of arachidonic acid was dissolved in methanol (10 mg/ml) and mixed with 5 μCi of ^{14}C-labeled arachidonic acid; and to obtain a dried substrate, the solvent was evaporated by blowing with nitrogen gas at room temperature. Subsequently, the substrate was dissolved in 50 μl of dimethylsulfoxide (DMSO), and 10 ml of 5 mM Tris-HCl buffer (pH 8.0) containing 2 mM phenol was added to the substrate solution, followed by sonication to completely dissolve the sample. Separately, test compounds having final concentrations of 0.1-10 μM were prepared. In this experiment, a human serum albumin (HAS)-binding compound was dissolved with the above buffer, and the other compounds were dissolved with DMSO. Five μl of each compound solution was added to 5 μl of the above substrate solution, followed by mixing. The samples were then preincubated at 35°C for about 10 min. Cyclooxygenase 2 derived from sheep placentae (Cayman Chem.) had been separately diluted 10-fold with the above buffer, and 20 μl of the enzyme solution was added to each of the above samples. The enzyme reaction was carried out at 35°C for 30 min, and stopped by adding 0.5 ml of ice-cold ethanol to each sample. Then, to 0.9 ml of each sample,

2.0 ml of 2% acetic acid solution was added. Arachidonic acid and its metabolite prostaglandin H2 were then extracted with 3.0 ml of ethyl acetate. From each extracted sample, a 2.0-ml portion was transferred to a new test tube. The solvent was distilled off from the transferred samples under reduced pressure, and the samples were dried *in vacuo*. Then each dried sample was dissolved in 100 µl of methanol, out of which 5 µl was subjected to analysis by high resolution thin layer chromatography. Arachidonic acid and its metabolite prostaglandin H2 were separated by chromatography with a developing solution of chloroform, ethyl acetate, methanol, acetic acid and water in proportions of 70:30:8:1:0.5 (v/v). The chromatography plate, together with a ^{14}C-standard substance, was exposed to an imaging plate (Fuji Film), and was analyzed with Fuji Film Bio-Image analyzer BAS-2000 to obtain an autoradiogram. By scanning of spots corresponding to the standard radioactive samples, a standard dose-response curve was plotted. Then, by use of the standard curve, each scanned value of arachidonic acid spots and prostaglandin H2 spots was converted to a amount of radiation. Indomethacin was purchased from Sigma, and PZ25 was a gift from Rhone-Poulenc Rorer.

The results are shown in Table 1.

Table 1. Cyclooxygenase Inhibitor 2

Dose of compound (μM)	Generated Amount of Prostaglandin H2 (%)			
	Compound A	Compound B	PZ25	Indomethacin
1	91.8±14.3	78.33±10.58	N.D.	N.D.
3	78.6±5.28	72.13±4.53*	103.25±9.29	95.55±7.87
10	46.88±6.49**	35.40±1.49**	103.53±11.2	99.45±7.80
30	20.10±1.95**	N.D.	93.9±9.06	96.55±9.63
	N.D.	N.D.	N.D.	N.D.

ND: Not done

Significance level for p-value: *, P<0.05; **, P<0.01 (calculated by Williams-Wilcoxon test)

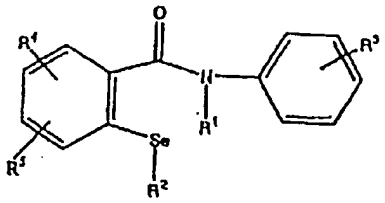
Compound A: 2-phenyl-1,2-benzisoselenazol-3(2H)-one

Compound B: S-(2- phenylcarbamoyl-phenylselenyl)albumin

PZ25: 2-phenyl-1,2-benzisothiazol-3(2H)-one

What is Claimed is:

1. A cyclooxygenase-2-inhibitor comprising, as an active ingredient, a compound represented by the following formula (1) or (1'):



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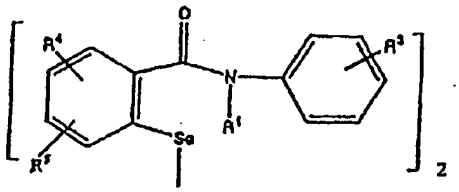

(1')

wherein R^1 represents a hydrogen atom or an alkyl group having 1-3 carbon atoms; R^2 represents a hydrogen atom, a hydroxyl group, an organic group capable of being bound through its thiol group within the molecule, or R^1 and R^2 may join to each other to form a single bond; R^3 represents a hydrogen atom, a halogen atom, an alkyl group having 1-3 carbon atoms, an alkoxyl group having 1-3 carbon atoms, a trifluoromethyl group, or a nitro group; each of R^4 and R^5, which may be identical to or different from each other, represents a hydrogen atom, a halogen atom, an alkoxyl group having 1-4 carbon atoms, a trifluoromethyl group, or R^4 and R^5 may join to each other to form a methylenedioxy group; a salt thereof, or a hydrate thereof.

2. The cyclooxygenase-2-inhibitor according to Claim 1, wherein R^2 is a peptide, a protein, or a glycoprotein, and is

capable of being bound through its thiol group within the molecule.

3. The cyclooxygenase-2-inhibitor according to Claim 1 or 2, wherein R^2 is albumin, a glutathione group, or an α-amino acid group, and is capable of being bound through its thiol group within the molecule.

4. The cyclooxygenase-2-inhibitor according to claim 3, wherein said albumin is a human albumin.

5. A cyclooxygenase-2-inhibitor comprising, as an active ingredient, 2-phenyl-1,2-benzisoselenazol-3(2H)-one, a salt thereof, or a hydrate thereof.

6. A cyclooxygenase-2-inhibitor comprising, as an active ingredient, S-(2-phenylcarbamoyl-phenylselenyl)-albumin, a salt thereof, or a hydrate thereof.

7. A method for producing a pharmaceutical composition for the therapy and/or prophylaxis of such diseases which are caused in a disturbance and/or in an influence of the cyclooxygenase-2-inhibition wherein for producing of said composition a cyclooxygenase-2-inhibitor according to one of the preceding claims 1 to 6 is used as active ingredient.

8. A method for producing a pharmaceutical composition for the therapy and/or prophylaxis of ischemic diseases, senile dementia, cancer, asthma, arterial sclerosis, and/or inflammation diseases wherein for producing of said composition

a cyclooxygenase-2-inhibitor according to one of the preceding claims 1 to 6 is used as active ingredient.

9. The method according to claim 7 or 8, wherein for producing of said composition a mixture of cyclooxygenase-2-inhibitors according to one of the preceding claims 1 to 6 is used as active ingredient.

10. The method according to one of the claims 7 to 9, wherein for producing of said composition beside the active ingredient or beside of the active ingredient mixture further at least one excipient, binder, disintegrator, and/or solubilizer is used.

11. The method according to one of the claims 7 to 10, wherein said composition is formulated in the form of a tablet, capsule, powder, granule, syrup, and/or a preparation for injection.

12. A method for therapy and/or prophylaxis of such diseases which are caused in a disturbance and/or in an influence of the cyclooxygenase-2-inhibition, wherein a pharmaceutical composition comprising a cyclooxygenase-2-inhibitor according to one of the preceding claims 1 to 6 as active ingredient is orally administered in a daily dosage of 100 to 2000 mg, relative to the active ingredient, for an adult.

13. The method according to claim 12, wherein said composition is orally administered in a daily dosage of 200 to 1000 mg, relative to the active ingredient, for an adult.

14. A method for therapy and/or prophylaxis of such diseases which are caused in a disturbance and/or in an influence of the cyclooxygenase-2-inhibition wherein a pharmaceutical composition comprising a cyclooxygenase-2-inhibitor according to one of the preceding claims 1 to 6 as active ingredient is perorally administered in a daily dosage of 0.05 to 1000 mg, relative to the active ingredient, for an adult.

15. A method for therapy and/or prophylaxis of ischemic diseases, senile dementia, cancer, asthma, arterial sclerosis, and/or inflammation diseases, wherein a pharmaceutical composition comprising a cyclooxygenase-2-inhibitor according to one of the preceding claims 1 to 6 as active ingredient is orally administered in a daily dosage of 100 to 2000 mg, relative to the active ingredient, for an adult.

16. The method according to claim 15, wherein said composition is orally administered in a daily dosage of 200 to 1000 mg, relative to the active ingredient, for an adult.

17. A method for therapy and/or prophylaxis of ischemic diseases, senile dementia, cancer, asthma, arterial sclerosis, and/or inflammation diseases, wherein a pharmaceutical composition comprising a cyclooxygenase-2-inhibitor according

to one of the preceding claims 1 to 6 as active ingredient is perorally administered in a daily dosage of 0.05 to 1000 mg, relative to the active ingredient, for an adult.

18. The method according to one of the claims 12 to 17, wherein said composition is administered in the form of a tablet, capsule, powder, granule, syrup, and/or a preparation for injection.

19. The method accoring to claim 18, wherein said composition is administered one time to three times each day.

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Veröffentlicht

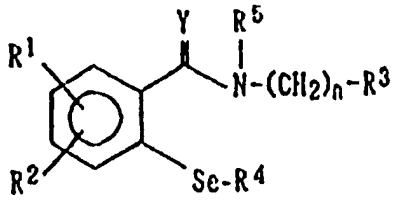
Ohne internationalen Recherchenbericht und erneut zu veröffentlichen nach Erhalt des Berichts.

(54) **Title:** THERAPEUTIC ANTI-ASTHMA AGENTS CONTAINING SELENIUM COMPOUNDS

(54) **Bezeichnung:** THERAPEUTISCHE MITTEL, ENTHALTEND ORGANISCHE SELENVERBINDUNGEN GENGEN ASTHMA

(57) **Abstract**

The description relates to a therapeutic agent which comprises as the active agent a compound given by the formula (1) and/or (1') and/or a pharmaceutically acceptable salt thereof. The agent of the invention exhibits great effectiveness in cases of both immediate and later asthmatic reactions, especially the latter. Owing to its low toxicity, the agent of the invention can safely be prescribed for human beings.



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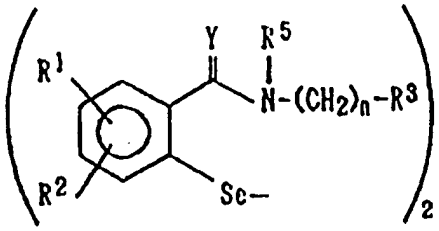

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(57) **Zusammenfassung**

Es wird ein therapeutisches Mittel beschrieben, das als Wirkstoff eine Verbindung, die durch die nachfolgende Formel (1) und/oder (1') wiedergegeben ist, und/oder ein pharmazeutisch akzeptables Salz dieser Verbindung umfaßt. Das erfindungsgemäße Mittel zeigt hohe Wirksamkeiten sowohl in bezug auf die unmittelbaren als auch späten asthmatischen Reaktionen und insbesondere auf die späten asthmatischen Reaktionen. Bedingt durch die geringe Toxizität kann das erfindungsgemäße Mittel sicher beim Menschen dargereicht werden.

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THERAPEUTISCHE MITTEL, ENTHALTEND ORGANISCHE SELENVERBINDUNGEN GENGEN ASTHMA

Die vorliegende Erfindung betrifft ein therapeutisches Mittel gegen Asthma, das wirksam asthmatische Reaktionen unterdrückt, insbesondere späte asthmatische Reaktionen beim Bronchialasthma.

Allgemein wurde erkannt, daß Bronchialasthma als eine Erkrankung gilt, die durch Kontraktion von weichen Muskeln im Luftweg aufgrund von Typ I Anaphylaxe verursacht wird. Jedoch haben kürzliche Erkenntnisse in diesem Forschungsgebiet aufgezeigt, daß ein Teil der asthmatischen Pathogenese dadurch verursacht wird, daß entgegengesetzte Luftbegrenzung, Luftwegentzündung, Mucus Hypersekretion und Neuausbildung der Luftwegstruktur in Folge der chronischen Entzündung ausgebildet werden. Von daher sollte eine pharmakologische Therapie über das Verständnis dieser Pathogenese hinaus dies entsprechend berücksichtigen.

Die zur Zeit verfügbaren Medikamente für Asthma sind Verringerungsmittel für die entgegengesetzte Luftbegrenzung, wie zum Beispiel Beta-Agonisten, und Kontrollmittel, so zum Beispiel Cortisoninhalationsmittel, zur Vermeidung von Symptomen durch Unterdrückung von Luftwegsentzündungen. Ein Beta-Agonist wirkt schnell in bezug auf eine Bronchialverengung und den damit verbundenen Begleitsymptomen, so zum Beispiel Husten, Brustenge und Keuchen. Ein inhaliertes Corticosteroid muß dauerhaft und täglich benutzt werden, um eine dauerhafte Kontrolle bei chronischem Asthma sicherzustellen.

Jedoch hat ein Beta-Agonist einige nachteilige Nebenwirkungen, wie zum Beispiel Stimulation der Herzgefäße, Muskelzuckungen und Hypokaliämie und Reizungen. Desweiteren verstärkt ein inhaliertes Cortisosteroid Candidiasis (oropharymgeal candidiasis), Dysphonie und gelegentliches Hu-

sten durch Reizung der oberen Luftwege, so daß das Risiko von systemischen Effekten eines Inhalationsmittels geringer ist als die systemischen Corticosteroide. Die lange Verwendung von oral oder parenteral applizierten Corticosteroiden kann ernsthafte Nebenwirkungen, wie zum Beispiel Osteoporose, Bluthochdruck, Diabetes, Hypophyseerkrankungen, grauer Star, Korpulenz, Muskelschwäche und Hautverdünnungen führen, die eine Hauterkrankung (cutaneous striae) und eine leichte Druckempfindlichkeit bewirken. Kontrollmittel werden für eine lange Zeit dargereicht und von daher sollten die systemischen Nebeneffekte von solchen Mitteln vermieden oder verringert werden.

Von dem Konzept, daß Kontrollmittel minimale Nebeneffekte mit Ausnahme in bezug auf die hauptsächlichen bronchialerweiternden oder entzündungshemmende Effekte besitzen, wurden antiallergische Mittel, wie beispielsweise Histamin-Rezeptor-Antagonisten, Leukotrien-Rezeptor-Antagonisten, Thromboxan-synthetase-Inhibitoren/Rezeptor-Antagonisten entwickelt. Jedoch gibt es nicht ausreichende Daten bezüglich ihrer klinischen Vorteile in der Langzeitbehandlung von Asthma und von daher wird die Entwicklung von höher wirksamen entzündungshemmenden Mitteln und weiteren klinischen Erprobungen erwartet.

Es wird angenommen, daß die auftretenden späten asthmatischen Reaktionen nach Reizung des Immunsystems mit einer Luftwegsentzündung begleitet ist. Zwei Arten der asthmatischen Reaktionen wurden unmittelbar nach der Antigenreizung und einige Stunden danach beobachtet. Die frühere Reaktion wird als unmittelbare asthmatische Reizung (IAR) und das darauf folgende Phänomen als späte asthmatische Reizung (LAR) bezeichnet. Die unmittelbare asthmatische Reizung wurde durch die Luftwegsbegrenzung, die von der akuten Bronchialverengung herrührt, hervorgerufen durch den Allergenausstoß, erkannt, während die späte asthmatische Reizung eine Luftwegsentzündung im Luftweg begründet. Die Luftwegsentzündung, die üblicherweise durch eine extensive Infiltration von Cosinophilen, Zellen und von einzelligen Zellen gekennzeichnet ist, bewirkt eine Schwellung der Luftwegswände mit oder ohne leichte

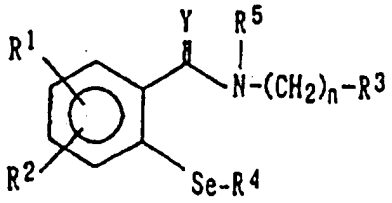
Muskelkontraktion. Derartige pathologische Änderungen werden nicht nur allein mit der späten asthmatischen Veränderung sondern auch mit der Luftwegshyperreaktivität und der Asthmaverschlimmerung in Verbindung gebracht (Metzger, W.J., Hunninghake, G.W. and Richardson, H.B.: Late Asthmatic Responses; Inquiry into Mechanism and Significance, Clin. Rev. Allergy 3:145, 1985). Der Mechanismus derartiger pathologischer Erscheinungen ist bis jetzt noch nicht vollständig erforscht.

Dementsprechend ist es erwünscht, ein Mittel zur Behandlung von Bronchialasthma zu entwickeln, das eine ausgezeichnete Wirksamkeit ähnlich zu der von Adrenal-Cortex-Hormonen besitzt, das gleichzeitig sowohl die unmittelbaren asthmatischen Reaktionen als auch die späten asthmatischen Reaktionen unterdrückt und das sicher ist.

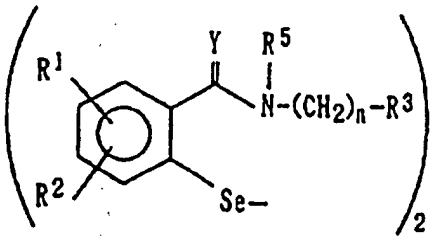
Es ist bekannt, daß 2-Phenyl-1,2-Benzoisoselenazole-3(2H)-one, das üblicherweise auch als Ebselen bezeichnet wird und das eine typische in der vorliegenden Erfindung verwendete Verbindung darstellt, eine deutliche Lipoxygenase inhibierende Wirkung besitzt (Peter Kuhl et al, Prostaglandins, 31 (1986), 1029-1048). Jedoch ist niemals darüber berichtet worden, daß Ebselen bei der Behandlung von Asthma wirksam ist.

Unter den vorstehend genannten Umständen haben die Erfinder ausführliche Studien durchgeführt und festgestellt, daß die Verbindungen, die nachfolgend noch durch die Formeln (1) und/oder (1') gekennzeichnet sind, eine hohe Wirksamkeit in bezug auf die Unterdrückung von asthmatischen Reaktionen, insbesondere in bezug auf späte asthmatische Reaktionen, bei Bronchialasthma besitzen.

Dementsprechend stellt die vorliegende Erfindung ein therapeutisches Mittel zur Behandlung von Asthma zur Verfügung, das als Wirksubstanz eine Verbindung der nachfolgenden Formel (1) und/oder der Formel (1') und/oder ein pharmazeutisch akzeptierbares Salz davon enthält.



Formel (1)



Formel (1')

In Formel (1) bzw. (1')

stellen R^1 und R^2 unabhängig voneinander ein Wasserstoffatom, ein Halogenatom, eine Trifluormethylgruppe, eine Nitrogruppe, eine C_1-C_6-Alkyl-Gruppe oder eine C_1-C_6-Alkoxyl-Gruppe dar, wobei R^1 und R^2 unter Ausbildung einer Methylendioxygruppe miteinander vernetzt sein können ;

R^3 ist eine Arylgruppe, eine aromatische heterocyclische Gruppe, eine 5 bis 7 Mitglieder enthaltende Cycloalkylgruppe oder eine 5 bis 7 Mitglieder enthaltende Cycloalkenylgruppe, wobei die Arylgruppe, die aromatische heterocyclische Gruppe, die Cycloalkylgruppe und/oder die Cycloalkenylgruppe substituiert sein kann;

R^4 stellt ein Wasserstoffatom, eine Hydroxylgruppe, eine -S-Glutathione-Gruppe, eine -S-α-Aminosäure-Gruppe oder eine Aralkyl (Arylalkyl)-Gruppe, die an ihrem Arylrest substituiert sein kann, dar;

R^5 ist ein Wasserstoffatom oder eine C_1-C_6-Alkylgruppe, wobei R_4 und R_5 unter Ausbildung einer einzigen Bindung miteinander verbunden sein können;

Y stellt ein Sauerstoffatom oder ein Schwefelatom dar und

n eine ganze Zahl zwischen 0 und einschließlich 5 ist, wobei das Selenatom oxidiert sein kann.

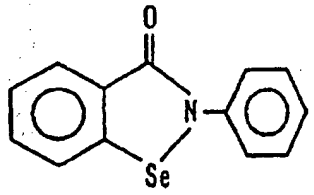
Die Figur 1 zeigt die Wirksamkeit des Unterdrückungseffektes von Ebselen bei asthmatischen Reaktionen bei Meerschweinchen, die durch Antigene induziertes Asthma erkrankt sind.

In der vorliegenden Erfindung umfassen die Mittel zur Behandlung von Asthma auch solche Wirkstoffe, die für die Behandlung von Asthma eingesetzt werden mit der Erwartung der Verbesserung von asthmatischen Bedingungen und vorbeugender Behandlung.

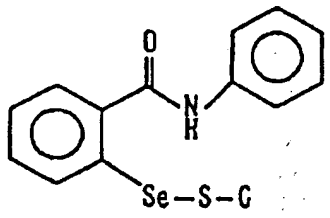
Verbindungen, die als Wirkstoffe in den Mitteln für die Behandlung von Asthma eingesetzt werden, werden durch die vorstehend wiedergegebenen Formeln (1) und (1') repräsentiert. Beispiele der bei R^1 aufgeführten C_1-C_6-Alkylgruppe umfassen die Methylgruppe, die Ethylgruppe, die Propylgruppe, die Isopropylgruppe, die n-Butylgruppe, die Isobutylgruppe, die sekundäre Butylgruppe und die Pentylgruppe. Beispiele der C_1-C_6-Alkoxyl-Gruppe umfassen die Methoxylgruppe, die Ethoxylgruppe und die Propoxylgruppe.

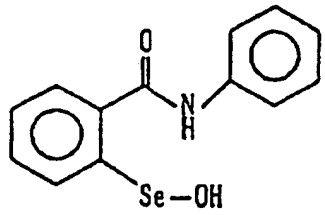
Beispiele der bei R^3 aufgeführten Arylgruppe umfassen die Phenylgruppe; Beispiele der Cycloalkyl-Gruppe umfassen die Cyclopentylgruppe, die Cyclohexylgruppe und die Cycloheptylgruppe; Beispiel der Cycloalkenylgruppe umfassen die 1-Cyclopentenylgruppe, 1-Cyclohexenylgruppe und 1-Cycloheptenylgruppe; und Beispiele der aromatischen heterocyclischen Gruppe umfassen die 5- oder 6-gliederigen aromatischen Heterocylengruppen, wie zum Beispiel die Pyridylgruppe, die Pyrimidylgruppe, die Imidazolylgruppe, die Oxazolylgruppe, die Isoxazolylgruppe, die Thiazolylgruppe und die Furylgruppe, wobei diese Gruppen substituiert sein können. Beispiele von Substituenten umfassen die C_1-C_6-Alkylgruppe, die C_1-C_6-Alkoxylgruppe, die Halogengruppe, die Carboxylgruppe

und die Hydroxygruppe. Die Zahl der Substituenten liegt vorzugsweise zwischen 1 und 3. Bei den R^4-Gruppen bezeichnet die -S-Glutathiongruppe eine Gruppe, die durch Entfernung eines Wasserstoffatoms von dem Thiolrest des Glutathions gebildet ist, während die -S-α-Aminosäuregruppe eine Gruppe anzeigt, die durch Entfernung eines Wasserstoffatoms vom Thiolrest der Thiol-enthaltenden α-Aminosäure gebildet wird. Beispiele der Arylalkylgruppe (Aralkyl) umfassen die Benzylgruppe. R^4 und R^5 bilden vorzugsweise eine einzige Bindung. Insbesondere umfaßt die vorliegende Erfindung die Verbindung 2-Phenyl-1,2-Benzoisoselenazole-3(2H)-one, das durch die folgende Formel wiedergegeben ist:

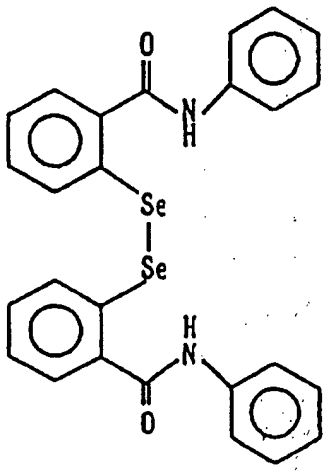


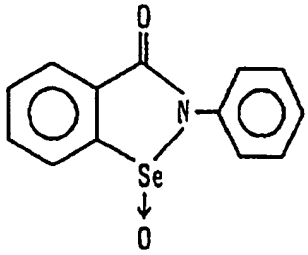
Die Verbindungen, die nachfolgend durch die folgenden Formeln wiedergegeben werden und die als aktive Metaboliten der zuvor wiedergegebenen Verbindung aufzufassen sind, sind ebenfalls bevorzugt.





(-S-G bezeichnet eine -S-Glutathin-Gruppe)





Darüber hinaus können in der vorliegenden Erfindung pharmazeutisch akzeptable Salze, die von den vorstehend aufgelisteten Verbindungen herstammen, verwendet werden.

Die vorstehend beschriebenen Verbindungen (1) oder (1') sind bereits bekannt. Sie können beispielsweise in Übereinstimmung mit dem Verfahren, wie dieses in den japanischen Offenlegungsschriften (kokai) Nr. 59-42373, 57-67568, 59-39894, 60-226868, 61-50963 oder in Biochemical Pharmacology Vol. 3, No. 20, 3235-3239 und 3241-3245 (1984) beschrieben sind, hergestellt werden.

Die Verbindungen (1) oder (1') und pharmazeutisch akzeptable Salze davon besitzen exzellente Wirksamkeiten in bezug auf die Unterdrückung von unmittelbaren oder späten asthmatischen Reaktionen bei Bronchialasthma, insbesondere bei späten asthmatischen Reaktionen, wie dies in den nachstehend wiedergegebenen Ausführungsbeispielen belegt wird.

Darüber hinaus konnte gefunden werden, daß die Verbindungen sehr sicher sind, wie dies durch die nachfolgenden und in der Tabelle 1 wiedergegebenen LD_{50}-Werte bewiesen wird, wobei diese Werte das Ergebnis eines Toxizitätstests unter Verwendung von Mäusen und Ratten, denen diese Verbindungen oral oder intraperitoneal verabreicht wurden, darstellen. Selbst dann, wenn diese Verbindungen in hohen Dosen verabreicht werden, zeigen sie keine Nebeneffekte, die ernsthafte Probleme verursachen.

Tabelle 1

Tier	Applikationsweg	LD_{50} (mg/kg)
Maus	p.o.	> 6810
	i.p.	740
Ratte	p.o.	> 6810
	i.p.	580

Die erfindungsgemäßen Mittel zur Behandlung von Asthma können in verschiedenen Formen von Zubereitungen für die orale oder parenterale Verwendung formuliert werden, so zum Beispiel als Tabletten, Kapseln, Puder, feine Granulate, Flüssigkeiten, Suspensionen, Emulsionen, Trockensirups, Inhalationsmittel, Injektionen und Zäpfchen. Derartige Zubereitungen werden nach den üblichen Methoden dadurch hergestellt, daß zu der Verbindung (1) oder (1') oder zu einem pharmazeutisch akzeptierbaren Salz davon Gleitmittel, Sprengmittel, Bindemittel oder Trägermittel zugesetzt werden.

Die Dosis der Verbindung (1), (1') oder des pharmazeutisch akzeptierbaren Salzes davon hängt von dem Applikationsweg, dem Zustand des Patienten, dem Körpergewicht des Patienten o. dgl. ab. Im Falle der oralen Darreichung variiert die Dosis üblicherweise bei einem Erwachsenen zwischen 100 bis 2.000 mg/Tag, vorzugsweise zwischen 200 bis 1.000 mg/Tag.

Wie bereits vorstehend beschrieben, unterdrücken die erfindungsgemäßen Mittel sowohl die unmittelbaren als auch die späten asthmatischen Reaktionen bei Bronchialasthma. Insbesondere bewirken die Mittel herausragende Effekte in bezug auf die späten asthmatischen Reaktionen. Darüber hinaus besitzen die erfindungsgemäßen Mittel eine geringe Toxizität und sind von daher sicher in bezug auf Menschen.

Die vorliegende Erfindung wird nachfolgend anhand von Ausführungsbeispielen näher erläutert, wobei die Ausführungsbeispiele die vorliegende Erfindung nicht einschränken sollen.

Testbeispiel

Die Wirksamkeiten von Ebselen wurden unter Verwendung von Meerschweinchen, die ein Antigen induziertes Asthma besitzen, durch Überprüfung der Lungenfunktionen unter Bewußtsein und spontaner Atmung getestet.

Verfahren:

1. Tiere

Jedes der weiblichen Hartley Meerschweinchen (SLC) (4 Wochen alt, Körpergewicht etwa 250 bis 300 g) wurden vorbehandelt mit Cyclophosmid (2 mg/kg). Nach zwei Tagen wurden intraperitoneal Ovalbumin (OA, 1 mg) und Aluminiumgel (1 g) injiziert. Drei Wochen später wurden wiederum OA (0,1 mg) und Aluminiumgel (1 g) intraperitoneal injiziert. Die so behandelten Meerschweinchen wurden als sensibilisierte Tiere benutzt. Zu diesem Testzeitpunkt waren die Meerschweinchen elf Wochen alt und hatten ein Körpergewicht von 450 bis 580 g.

2. Apparate und Werkzeuge

Körper-Pressmograph, Drucktyp
Pulmotacograph (TP-601G, Nippon Kodensha)
Differentialmeßwandler (T-601, Nippon Kodensha)
Gasflußwiderstandsrohr (Lilly type, Nippon Kodensha)
Oszilloskop (DS-9121, Iwasaki Tsushinki)
Computer (Macintosh Centris 660 AV, Apple)
Software (zur Analyse der Atmung basierend auf LabView für Macintosh 3.01)
Zerstäuber (NE-U10, Tateishi Denki)

3. Verfahren zur Darreichung der Mittel und Induzierung der Antigene

Gruppe von mit Dexamethason vorbehandelten Meerschweinchen

Dexamethason (1 mg/kg) wurde in 100 % DMSO (1 ml) gelöst. Diese Lösung wurde während vier aufeinander folgende Tage intraperitoneal injiziert. Die letzte intraperitoneale Injektion wurde 24 Stunden vor der Induzierung der Antigene durchgeführt.

Gruppe der mit Ebselen vorbehandelten Meerschweinchen

Ebselen (5 mg/kg) wurde in 100 % DMSO (1 ml) gelöst. Diese Lösung wurde intraperitoneal 30 Minuten vor der Induzierung der Antigene injiziert.

Kontrollgruppe

100 % DMSO (1 ml) wurde intraperitoneal 30 Minuten vor der Induzierung der Antigene injiziert.

Verfahren zur Induzierung von Antigenen

OA (40 mg) wurde in einer physiologischen Salzlösung (4 mg/ml) gelöst. Diese Lösung wurde 2 Minuten lang unter Verwendung eines Ultraschallzerstäubers inhaliert.

4. Verfahren zur Überprüfung der Lungenfunktion

In Übereinstimmung mit dem Verfahren von Agrawal (Agrawal, K. P.; Specific Airway Conductance in Guinea Pigs: "Normal Values and Histamine Induced Fall", Respiratory Physiology 43:23, 1981) wurde jedes Meerschweinchen auf einem Körper-Pressmograph, Drucktyp, fixiert und die prozentuale Variation des spezifischen Luftweg-Leitwertes (sGaw) wurde gemessen. Der Luftfluß durch die Nase und die Änderung des Innendruckes der Box wurden aufgezeichnet. Die entsprechenden Wellenformen wurden digital bei 1.024 Hz gesammelt und die Punkte vom Ende des Ausatmens bis zum Beginn des Einatmens wurden als rückläufige Linie aufgezeichnet. Unter Verwendung der Neigung (tan) der rückläufigen Linie wurde der sGaw erhalten. Der sGaw-Wert wurde vor der Induzierung der Antigene gemessen. Nachdem eine physiologische Salzlösung für zwei Minuten inhaliert wurde, wurde der sGaw nochmals gemessen, um sicherzustellen, daß sich keine Daten geändert hatten. Der zu diesem Zeitpunkt gemessene Wert wurde als 100 % angenommen und die prozentuale Variation des sGaw wurde nach Induzierung der Antigene bestimmt.

Die Ergebnisse sind in Figur 1 dargestellt. Bei der Gruppe von Meerschweinchen, die mit Ebselen vorbehandelt wurden, wa-

ren sowohl die unmittelbaren als auch die späten asthmatischen Reaktionen wirkungsvoller unterdrückt als bei den Meerschweinchen der Kontrollgruppe.

Wenn die mit Ebselen vorbehandelte Gruppe mit der mit Dexamethason vorbehandelten Gruppe verglichen wird, sind die Bereiche der Unterdrückung der unmittelbaren asthmatischen Reaktionen überwiegend gleich. Jedoch wurden bei den mit Ebselen vorbehandelten Gruppen die späten asthmatischen Reaktionen erheblich und vollständig unterdrückt, während bei der Kontrollgruppe die späten asthmatischen Reaktionen zwischen 180 und 420 Minuten beobachtet wurden.

In diesem Zusammenhang ist darauf hinzuweisen, daß die Dexamethason-Dosis von 1 mg/kg, die über 4 Tage den Meerschweinchen verabreicht wurde, ungefähr einer Dexamethason-Dosis für einen Menschen (Körpergewicht 60 kg) von 240 mg (60 mg x 4 Tage) entspricht. Diese Dexamethason-Dosis entspricht 2.400 mg von Prednisolon (0,5 mg Dexamethason ist äquivalent zu 5 mg Prednisolon).

Diese Menge von Prednisolon (2.400 mg) entspricht der Menge, die in einer Pulstherapie verbraucht wird, in der 1 g/Tag Prednisolon in Folge während 3 Tage dargereicht wird.

Die vorstehenden Ausführungen beweisen eindeutig, daß Ebselen eine höhere Wirksamkeit im Vergleich zu solchen Therapien besitzt, die in früheren klinischen Situation angewendet wurden.

Beispiel 1

Tabletten

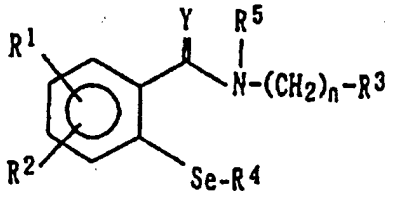
Unter Verwendung des bekannten Verfahrens wurden Tabletten der nachfolgenden Zusammensetzung hergestellt:

Ebselen	50 mg
Carboxymethylcellulose	25 mg

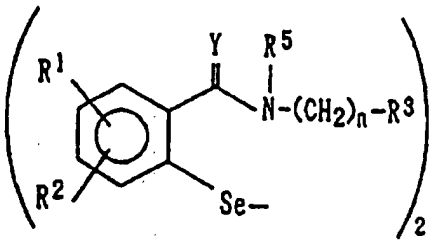
Stärke	5 mg
kristalline Cellulose	40 mg
Magnesiumstearat	2 mg
Summe	122 mg

Zusammenfassend ist festzuhalten, daß die vorliegende Erfindung ein sicheres therapeutisches Mittel zur Behandlung von Asthma, das sowohl die unmittelbaren als auch die späten asthmatischen Reaktionen bei Bronchialasthma unterdrückt, beschreibt.

Das erfindungsgemäße therapeutische Mittel umfaßt als Wirkstoff eine Verbindung, die durch die nachfolgende Formel (1) und/oder (1') wiedergegeben ist,



Formel (1)

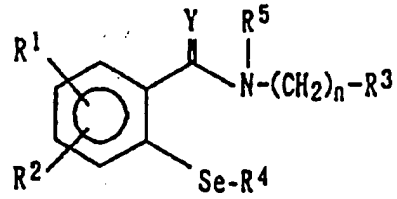


Formel (1')

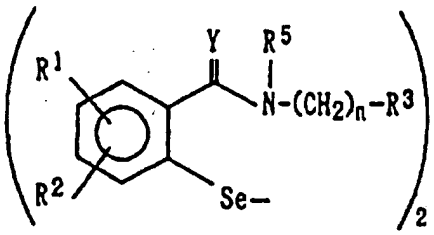
und/oder ein pharmazeutische akzeptables Salz dieser Verbindung. Das erfindungsgemäße Mittel zeigt hohe Wirksamkeiten sowohl in bezug auf die unmittelbaren als auch späten asthmatischen Reaktionen und insbesondere auf die späten asthmatischen Reaktionen. Bedingt durch die geringe Toxizität kann das erfindungsgemäße Mittel sicher beim Menschen dargereicht werden.

Patentansprüche

1. Therapeutisches Mittel gegen Asthma, dadurch gekennzeichnet, daß das therapeutische Mittel als Wirkstoff eine Verbindung, die durch die nachfolgenden Formeln (1) und/oder (1') wiedergegeben ist, und/oder ein pharmazeutisch akzeptables Salz davon, enthält,



Formel (1)



Formel (1')

wobei in Formel (1) bzw. (1')

R^1 und R^2 unabhängig voneinander ein Wasserstoffatom, ein Halogenatom, eine Trifluormethylgruppe, eine Nitrogruppe, eine C_1-C_6-Alkyl-Gruppe oder eine C_1-C_6-Alkoxyl-Gruppe darstellen, wobei R^1 und R^2 unter Ausbildung einer Methylendioxygruppe miteinander vernetzt sein können ;

R^3 eine Arylgruppe, eine aromatische heterocyclische Gruppe, eine 5 bis 7 Mitglieder enthaltende Cycloalkylgruppe oder eine 5 bis 7 Mitglieder enthaltende Cycloalkenylgruppe ist, wobei die Arylgruppe, die aromatische heterocyclische Gruppe, die Cycloalkylgruppe und die Cycloalkenylgruppe substituiert sein kann;

R^4 ein Wasserstoffatom, eine Hydroxylgruppe, eine -S-Glutathione-Gruppe, eine -S-α-Aminosäure-Gruppe, oder eine Aralkyl (Arylalkyl)-Gruppe, die an ihrem Arylrest substituiert sein kann, darstellt;

R^5 ein Wasserstoffatom oder eine C_1-C_6-Alkylgruppe ist, oder wobei R_4 und R_5 unter Ausbildung einer einzigen Bindung miteinander verbunden sein können;

Y ein Sauerstoffatom oder ein Schwefelatom darstellt,

n eine ganze Zahl zwischen 0 und einschließlich 5 ist und

das Selenatom oxidiert sein kann.

2. Therapeutisches Mittel nach Anspruch 1, dadurch gekennzeichnet, daß es als Wirkstoff 2-Phenyl-1,2-Benzoisoselenazole-3(2H)-one oder ein pharmazeutisch akzeptables Salz davon enthält.

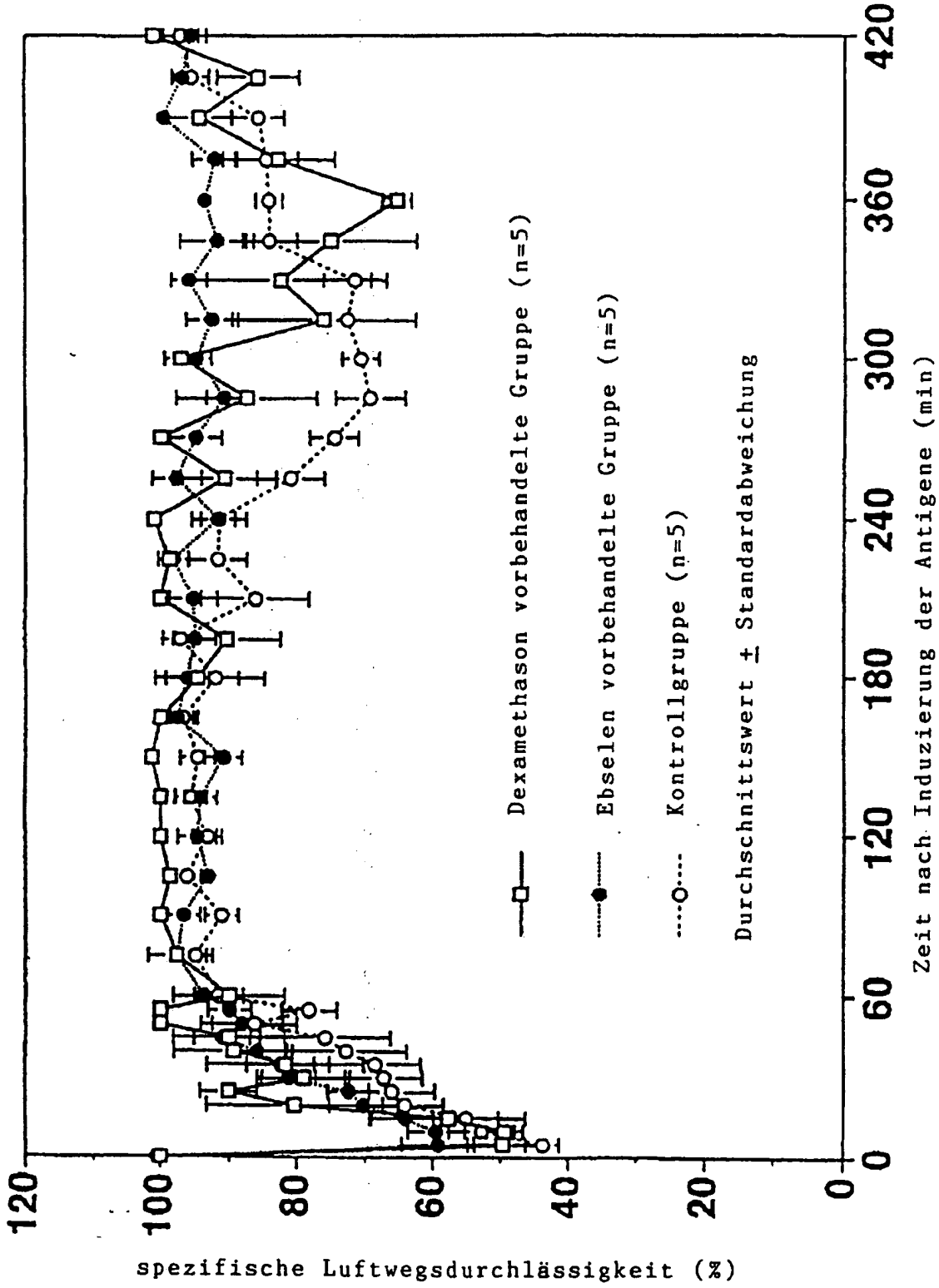
3. Therapeutisches Mittel nach Anspruch 1 zur Behandlung von Bronchialasthma.

4. Therapeutisches Mittel nach Anspruch 2 zur Behandlung von Bronchialasthma.

5. Therapeutisches Mittel nach Anspruch 1, dadurch gekennzeichnet, daß es die späten asthmatischen Reaktionen unterdrückt.

6. Therapeutisches Mittel nach Anspruch 2, dadurch gekennzeichnet, daß es die späten asthmatischen Reaktionen unterdrückt.

Fig 1


spezifische Luftwegsdurchlässigkeit (%)
120
100
80
60
40
20
0
0
60
120
180
240
300
360
420
Zeit nach Induzierung der Antigene (min)
Dexamethason vorbehandelte Gruppe (n=5)
Ebselen vorbehandelte Gruppe (n=5)
Kontrollgruppe (n=5)
Durchschnittswert ± Standardabweichung

Chem. Res. Toxicol. **1999**, *12*, 264−269

Function of Thioredoxin Reductase as a Peroxynitrite Reductase Using Selenocystine or Ebselen

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The activity of mammalian thioredoxin reductase as a peroxynitrite reductase was investigated. Peroxynitrite was infused to maintain a 0.2 μM steady-state concentration in potassium phosphate buffer (pH 7.4). Benzoate hydroxylation and nitrite formation were used as indices of oxidation reactions of peroxynitrite and of peroxynitrite reduction, respectively. In the presence of NADPH (10 μM), thioredoxin reductase at 50 nM alone did not significantly scavenge peroxynitrite, as shown by there being no significant effect on benzoate hydroxylation or nitrite formation. However, when selenocystine (1 μM) or ebselen (2 μM) was present in the reaction mixture, there was significant suppression of benzoate hydroxylation and an increase in nitrite formation until all the NADPH was oxidized. The addition of thioredoxin did not enhance these effects. In contrast, peroxynitrite reduction by ebselen complexed with BSA was enhanced by the presence of thioredoxin. In parallel experiments, thioredoxin reductase efficiently reduced ebselen selenoxide back to ebselen.

Introduction

Peroxynitrite[1] is a potent oxidizing and nitrating species. Tissue damage in a number of pathological conditions in humans and in experimental animals have been linked to in vivo peroxynitrite formation (see ref *1* for review). Selenium-containing amino acids and low-molecular weight organoselenium compounds (e.g., ebselen) rapidly react with peroxynitrite (*2*, *3*) and protect against DNA damage caused by peroxynitrite (*4*, *5*). We proposed that cellular proteins containing seleno-amino acids may selectively protect against damage due to peroxynitrite (*6*). Glutathione peroxidase can act as a peroxynitrite reductase (*7*), and recent work showed that selenoprotein P in human plasma may also act to protect against peroxynitrite (*8*).

Thioredoxin reductase (TR[2]), coupled with thioredoxin (Trx) and NADPH, is an efficient general protein disulfide reductase (see ref *9* for review). Mammalian TR has a much broader substrate specificity than its *Escherichia coli* counterpart, including selenite (*10*) and a number of organoselenium compounds [e.g., selenocystine (*11*)], as well as cytosolic and plasma glutathione peroxidases (*12*). In addition to efficiently reducing selenoproteins and selenocompounds, mammalian TR is itself a selenoprotein (*13*), containing a selenocysteine residue near the carboxy terminus (*14*). Here we investigated whether mammalian TR can function as a peroxynitrite reductase.

Experimental Procedures

Reagents. Diethylenetriaminepentaacetic acid (DTPA), selenocystine, *N*-(1-naphthyl)ethylenediamine, sulfanilamide, bovine serum albumin, and benzoic acid were from Sigma (Deisenhofen, Germany). MnO_2 was from Fluka (Buchs, Switzerland). Human Trx and bovine TR (EC 1.6.4.5) were from Imco (Stockholm, Sweden). NADPH was from Boehringer (Mannheim, Germany). 2-Phenyl-1,2-benzioselenazol-3(2*H*)-one (ebselen) was kindly provided by Rhône-Poulenc-Rorer (Cologne, Germany). Peroxynitrite was synthesized from sodium nitrite and H_2O_2 using a quenched-flow reactor (*15*), and H_2O_2 was eliminated by passing the peroxynitrite solution over MnO_2 powder. The final peroxynitrite concentration was determined spectrophotometrically at 302 nm (ϵ = 1700 M^{-1} cm^{-1}).

BSA−Ebselen Complex. An aqueous solution of BSA (580 μM) was mixed 1:1 (v/v) with dimethylformamide containing 4 mM dissolved ebselen or dimethylformamide alone (BSA control) and the mixture incubated for 15 min at 37 °C (*16*). Unbound ebselen was removed by passing the mixture through a Sephadex G-15 column (10 mm × 100 mm). Aliquots containing protein were pooled and then dialyzed against 1000 volumes of ddH_2O for 2 h at 4 °C, and then against 1000 volumes of PBS (pH 7.4, 4 °C) overnight. The absorption spectrum of the complex was used to determine the bound ebselen concentration (ϵ_{330} = 7700 M^{-1} cm^{-1}). The ratio of ebselen to BSA was calculated to be 0.3 mol per mole of BSA.

Hydroxylation of Benzoate Caused by Steady-State Infusion of Peroxynitrite. Peroxynitrite-mediated oxidation reactions were monitored using benzoate as a model target compound as described previously (*7*, *17*). Peroxynitrite was infused with a micropump at a rate of 35 μL/min from a 72 μM stock solution while being constantly mixed with a magnetic stirrer at room temperature into a mixture (0.4 mL) containing benzoic acid (1 mM) and DTPA (0.1 mM) in 0.5 M potassium phosphate buffer (pH 7.4); the final volume was 470 μL. The steady-state input concentration of peroxynitrite (0.2 μM) was calculated by using the infusion rate of peroxynitrite and its decay rate in phosphate buffer at 25 °C and pH 7.4 (0.41 s^{-1}). Hydroxylation of benzoate was determined fluorometrically (λ_{ex} = 300 nm, λ_{em} = 410 nm) 0, 0.5, 1, and 2 min after

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[1] The term peroxynitrite is used sometimes to refer to both the peroxynitrite anion proper ($ONOO^-$) and peroxynitrous acid (ONOOH); the IUPAC names are oxoperoxonitrate(1-) and hydrogen oxoperoxonitrate, respectively.

[2] Abbreviations: DTPA, diethylenetriaminepentaacetic acid; ebselen, 2-phenyl-1,2-benzioselenazol-3(2*H*)-one; BSA, bovine serum albumin; TR, thioredoxin reductase; Trx, thioredoxin.

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peroxynitrite infusion with a fluorescence microcuvette. Aliquots (15 μL) of the solutions were also taken for nitrite determination. Under these conditions, the individual and combined effects of selenocystine (1 μM), ebselen (2 μM), NADPH (10 μM), TR (50 nM), and Trx (300 nM) were determined relative to the effect of peroxynitrite alone (No addition). In control, reverse-order experiments, the various compounds used in this study did not significantly interfere with fluorescence determination of benzoate hydroxylation.

Formation of Ebselen Selenoxide and Reduction by TR. Ebselen (100 μM in 0.1 M potassium phosphate buffer with 5% methanol) was oxidized to ebselen selenoxide by bolus addition of peroxynitrite (200 μM), as described previously (*3*). To the resultant solution of ebselen selenoxide were added 50 nM TR and NADPH (100 μM); aliquots were removed at 0, 0.25, 0.5, 1, 2, and 4 min and added to 3 volumes of acetonitrile. Concentrations of ebselen and ebselen selenoxide in the organic phase of the solution were then determined by HPLC (see below).

HPLC Analysis. Ebselen and ebselen selenoxide were separated and detected by HPLC as described previously (*3*) with minor modifications. Aliquots of samples (50 μL) were injected onto a C-18 reverse-phase column (150 mm × 4.6 mm). Separation was performed with a 10 mM sodium phosphate buffer (pH 7.4)/acetonitrile gradient on a Merck-Hitachi L-6200A HPLC unit, at a flow rate of 1.0 mL/min. The linear gradient was from 90:10 to 24:76 over the course of 15 min. The organoselenium compounds were monitored with a Merck-Hitachi 655A UV detector equipped with a D-2500 Chromato-Integrator at 254 nm. Calibration curves were calculated from peak areas versus concentration values of standards.

Nitrite concentrations in samples were determined with the Griess reaction modified for HPLC detection. Briefly, Griess reagents [2.5 μL of 0.1% *N*-(1-naphthyl)ethylenediamine and 10 μL of 2.5% sulfanilamide in 15% phosphoric acid] were added to 37.5 μL of a diluted sample or standard, and the mixture was incubated for 3 min; then the entire mixture was injected onto a C-18 reverse-phase column (250 mm × 4 mm). Separation was performed with an aqueous solution with 40% methanol and 3% phosphoric acid with a flow rate of 1.0 mL/min. The resultant product was detected colorimetrically by its absorbance at 540 nm. The peak areas of nitrite standards were used to calculate standard curves.

Determination of NADPH. In experiments parallel to those described above for hydroxylation of benzoate, the amount of NADPH in the samples was monitored fluorometrically (λ_{ex} = 340 nm, λ_{em} = 450 nm) and compared with standards. These experiments were performed in the absence of benzoic acid to avoid possible interference with NADPH fluorescence.

Results

Protection by TR with Selenocystine or Ebselen against Hydroxylation of Benzoate during Steady-State Infusion of Peroxynitrite. In the experiments whose results are depicted in Figure 1A and summarized in Table 1, benzoate hydroxylation increased linearly with time when, over 2 min, peroxynitrite was infused with a micropump to maintain a steady-state concentration of 0.2 μM (Figure 1A, top curve, ●). Under these conditions, addition of 1 μM selenocystine (Figure 1A, ■), 300 nM thioredoxin, or 50 nM TR alone decreased benzoate hydroxylation by less than 15%. Addition of 10 μM NADPH prevented benzoate hydroxylation by 35% after 2 min of peroxynitrite infusion (Figure 1A, ▲). The combined effect of NADPH with TR in the presence (▼) and absence (◆) of Trx was additive under these conditions. However, when selenocystine was added to NADPH and TR, benzoate hydroxylation was completely prevented during the first minute of peroxynitrite infusion (Figure 1A, ◇); similar effects were observed when Trx (0.3 μM) was added (Figure 1A, ▽).

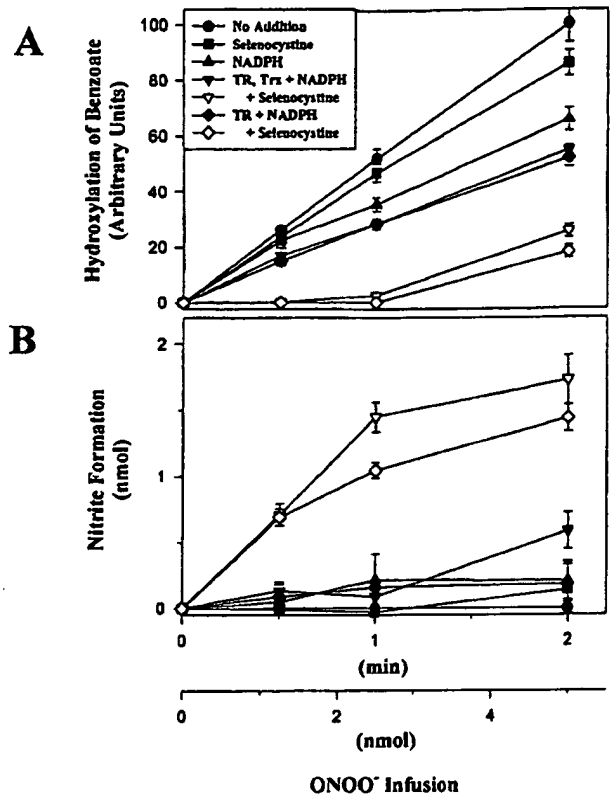

Figure 1. Thioredoxin system and selenocystine and peroxynitrite-induced hydroxylation of benzoate or nitrite formation during steady-state infusion. Panel A shows the course of peroxynitrite-mediated hydroxylation of benzoate in vitro as described in Experimental Procedures. Panel B shows the course of nitrite formation in the same samples as measured by Griess reaction modified for detection by HPLC as described in Experimental Procedures. The effects of selenocystine (1 μM) and NADPH (10 μM), alone or in combination with thioredoxin reductase (TR; 50 nM) and/or thioredoxin (Trx; 300 nM), are shown (see the key in the figure) and compared with the effect of peroxynitrite alone (No Addition). Results are means ± SEM; $n = 3-5$.

In the absence of NADPH, ebselen protected against peroxynitrite during the first 30 s of peroxynitrite infusion (Figure 2A, ▲). Since the ratio of ebselen to peroxynitrite during the first 30 s was about 1:1, ebselen was most likely oxidized completely by peroxynitrite (*3*). Addition of TR to ebselen had no effect under these conditions (▼). When NADPH and ebselen were combined, the effect was additive (◆). Analogous to the effect described above for selenocystine (Figure 1A), TR, ebselen, and NADPH together completely prevented benzoate hydroxylation during the first minute of peroxynitrite infusion (Figure 2A, ■). Similar results were observed when Trx (0.3 μM) was added (data not shown).

Nitrite Formation during Peroxynitrite Infusion. As the spontaneous decay of peroxynitrite generates nitrate, an increase in the yield of nitrite at the expense of nitrate measures peroxynitrite reduction (*7*). In our experiments, the basal nitrite level after spontaneous decay of 5.0 nmol of peroxynitrite infused during 2 min was 3.5 nmol, most likely due to synthesis or storage of peroxynitrite. Nitrite formation was calculated as increases over the spontaneous generation of nitrite during the decay of peroxynitrite. While NADPH alone decreased

Table 1. Effect of the Thioredoxin System and Selenocystine on Peroxynitrite-Induced Hydroxylation of Benzoate and Nitrite Formation during Steady-State Infusion[a]

	effect of peroxynitrite infusion (2 min)	
treatment	benzoate hydroxylation (arbitrary units)	amount of nitrite formed (nmol)
no addition[b,c]	100.0 ± 6.7	0.0
thioredoxin reductase (50 nM)	87.2 ± 5.1	0.1 ± 0.1
thioredoxin (300 nM)	95.9 ± 3.2	0.2 ± 0.2
selenocystine (1 μM)[b]	85.8 ± 4.5	0.1 ± 0.2
NADPH (10 μM)[b]	65.6 ± 4.1	0.1 ± 0.1
TR, Trx, and NADPH[b]	54.9 ± 1.6	0.6 ± 0.1
with selenocystine[b]	25.8 ± 2.3	1.7 ± 0.2
TR and NADPH[b]	51.9 ± 3.0	0.2 ± 0.1
with selenocystine[b]	18.2 ± 2.3	1.4 ± 0.1
Trx and NADPH	56.9 ± 5.1	0.3 ± 0.1
with selenocystine	57.0 ± 5.1	0.2 ± 0.2
TR and Trx	82.6 ± 5.1	0.1 ± 0.2
with selenocystine	72.6 ± 4.3	0.1 ± 0.1

[a] Peroxynitrite was infused as described in Experimental Procedures. The hydroxylation of benzoate and nitrite formation after 2 min of peroxynitrite infusion (5 nmol) was determined as described in Experimental Procedures. Results are means ± SEM; $n = 3-5$. [b] See Figure 1 for the time course during 2 min of $ONOO^-$ infusion. [c] The yield of product was determined to be 0.4% (~45 nM) relative to the amount of peroxynitrite infused.

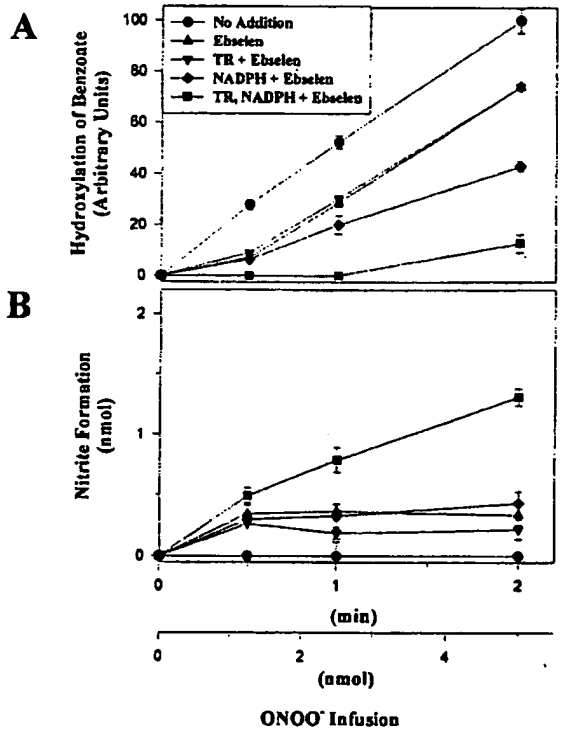

Figure 2. Thioredoxin system and ebselen and peroxynitrite-induced hydroxylation of benzoate or nitrite formation during steady-state infusion. Conditions were as described in Experimental Procedures and are analogous to those described in the legend of Figure 1. Panel A shows the course of peroxynitrite-mediated hydroxylation of benzoate, while panel B shows the course of nitrite formation in the same samples. The effects of ebselen (2 μM) alone or in combination with NADPH (10 μM) and thioredoxin reductase (TR; 50 nM) are shown (see the key in the figure) and compared with the effect of peroxynitrite alone (No Addition). Results are means ± SEM; $n = 3-5$.

benzoate hydroxylation by 35% (Figure 1A, ▲), no significant increase in nitrite formation was observed (Figure 1B, ▲), suggesting that NADPH does not directly react with peroxynitrite, but competes with benzoate for an oxidizing species of peroxynitrite. Selenocystine alone had no effect on nitrite formation (Figure 1B and Table 1). Ebselen (2 μM) alone did increase nitrite formation by 0.3 nmol during the first 30 s of peroxynitrite infusion (Figure 2B, ▲), as expected from previous studies (*3*). However, ebselen did not significantly increase nitrite formation during the subsequent 1.5 min of peroxynitrite infusion, further suggesting that ebselen is oxidized completely by peroxynitrite by the first 30 s of the experiment. Under conditions when a catalytic inhibition of benzoate hydroxylation was observed, i.e., when selenocystine or ebselen was combined with TR and NADPH (Figures 1A and 2A), a significant increase in nitrite formation was observed relative to control conditions (Figure 1B, ◇ and ▽; Figure 2B, ■).

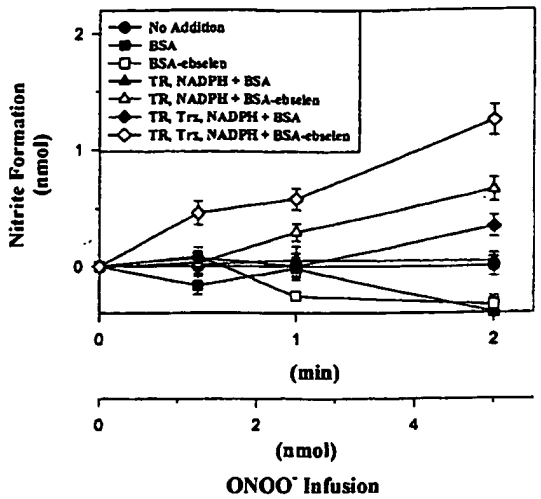

Figure 3. Thioredoxin system, BSA, and the BSA–ebselen complex and peroxynitrite-induced nitrite formation during steady-state infusion. Conditions were as described in Experimental Procedures. The effects of BSA (6 μM) and the BSA–ebselen complex (6 μM) alone or in combination with NADPH (10 μM), thioredoxin reductase (TR; 50 nM), and thioredoxin (Trx; 2.5 μM) on peroxynitrite-mediated nitrite formation are shown (see key on figure), and compared with the effect of peroxynitrite alone (No Addition). Results are means ± SEM; $n = 3-5$.

Nitrite tended to form more rapidly in the first minute than during the second minute. This effect was more pronounced with selenocystine (Figure 1B, ◇) than with ebselen (Figure 2B, ■). The ratio of the amount of nitrite formed to the amount of peroxynitrite infused during the first minute was ~0.4 and 0.3 for selenocystine and ebselen, respectively. Trx (300 nM) slightly increased nitrite formation during the first minute with selenocystine (Figure 1B, ▽) with a ratio of 0.6 mol of nitrite formed for each mole of peroxynitrite infused, but had no effect on nitrite release caused by ebselen (data not shown). Nitrite release with higher concentrations of TR (300 nM) alone with NADPH was similar to that of 50 nM TR and not significantly greater than controls (data not shown).

The production of nitrite by the thioredoxin system in the presence of BSA or BSA–ebselen was also investigated (Figure 3). Under these conditions, BSA–ebselen (6 μM protein concentration, □) or BSA (6 μM, ■) did not increase nitrite release compared to peroxynitrite alone ( ). Indeed, after 2 min of peroxynitrite infusion, nitrite formation decreased by 0.3 nmol. While the free cysteine of BSA can directly scavenge peroxynitrite with an

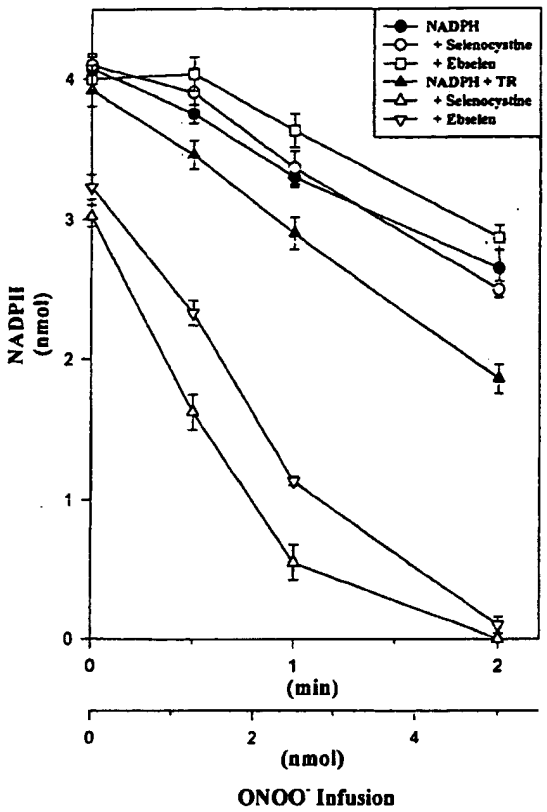

Figure 4. Effect of TR, selenocystine, and ebselen on the peroxynitrite-induced oxidation of NADPH during steady-state infusion. The oxidation of NADPH during infusion of peroxynitrite was monitored fluorometrically as described in Experimental Procedures. The effects of selenocystine (1 μM) and ebselen (2 μM) alone or in combination with thioredoxin reductase (TR; 50 nM) are shown (see the key in the figure) and compared with the effect of peroxynitrite alone (No Addition). If vehicle (0.1% KOH) was infused instead of peroxynitrite, the rate of NADPH oxidation was <0.15 nmol/min. Results are means ± SEM; $n = 3-5$.

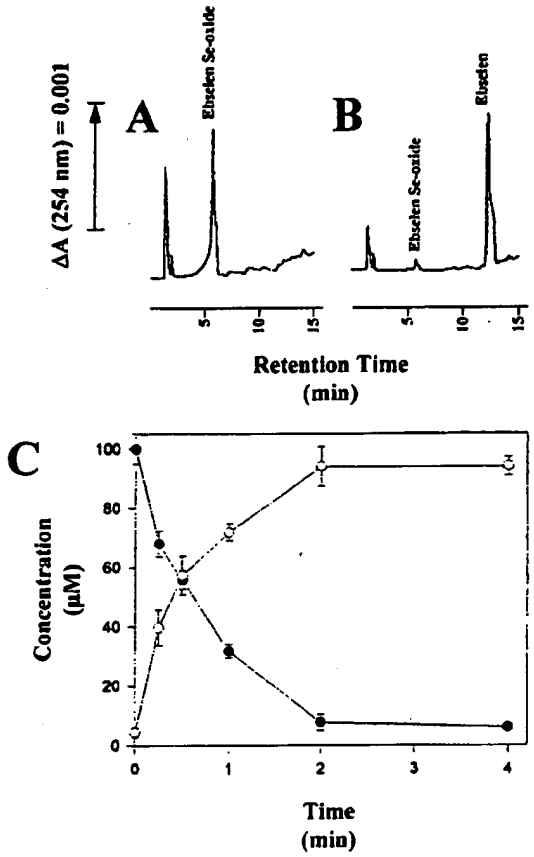

Figure 5. Formation of ebselen selenoxide by peroxynitrite and reduction by TR. Ebselen and ebselen selenoxide were detected by HPLC as described in Experimental Procedures. Ebselen selenoxide (100 μM) was formed by bolus addition of peroxynitrite (200 μM) under constant vortexing (A). Panel B shows the formation of ebselen after incubation of ebselen selenoxide (100 μM) with TR (50 nM) and NADPH (100 μM) for 2 min, and panel C gives the time course of the reduction of ebselen selenoxide (●) to ebselen (○) by TR and NADPH. Results are representative HPLC chromatographs (panels A and B) or means ± SEM; $n =$ 3–4 (panel C).

apparent second-order rate constant of 2800 M^{-1} s^{-1} at 37 °C (*18*), concentrations used here were too low to have a significant scavenging effect. While BSA, combined with TR and NADPH (▲), did not increase nitrite formation, BSA–ebselen, TR, and NADPH (△) did. The addition of Trx (2.5 μM) to the reaction mixture caused an increase in nitrite formation for both BSA (◆) and BSA–ebselen (◇) in the presence of TR and NADPH. However, while the effect with BSA was slight and only occurred after 2 min of peroxynitrite infusion (◆), the effect with BSA–ebselen (◇) occurred as early as after 30 s of peroxynitrite infusion; at this time point, the ratio of the amount of nitrite formed to the amount of peroxynitrite infused was 0.4.

Oxidation of NADPH during Peroxynitrite Infusion. The oxidation of NADPH during peroxynitrite infusion was observed in experiments parallel to those described for ebselen and selenocystine (Figures 1 and 2). The rate of NADPH oxidation when vehicle (0.1% KOH) was infused instead of peroxynitrite was <0.15 nmol/min under all conditions. Selenocystine (Figure 4, ○) had no significant effect on the oxidation of NADPH caused by peroxynitrite (●), while ebselen prevented NADPH oxidation during the first 30 s of peroxynitrite infusion (□); after the first 30 s, the rate of NADPH oxidation was similar for all three groups. In the presence of TR, the rate of NADPH oxidation was slightly higher (1.0 nmol/min, ▲) than with NADPH alone. When selenocystine or ebselen was included with TR (△ and ▽, respectively), basal NADPH concentrations decreased about 1 nmol prior to peroxynitrite infusion, and the rate of NADPH oxidation during peroxynitrite infusion was significantly increased (>2.5 nmol/min). If it is assumed that all NADPH is oxidized by reduction of selenocystine or ebselen after the reaction with peroxynitrite, the stoichiometry of the reaction is 1 mol of NADPH consumed per mole of peroxynitrite infused. Trx had no significant effect on the results described above (data not shown).

Reduction of Ebselen Selenoxide by TR. Figure 5 shows the reduction of ebselen selenoxide to ebselen by TR and NADPH. As observed previously (*3*), ebselen was efficiently oxidized to ebselen selenoxide by peroxynitrite (Figure 5A). Addition of TR (50 nM) and NADPH (100 μM) led to a rapid disappearance of ebselen selenoxide concomitant with an increase in the level of ebselen (Figure 5B). The amount of ebselen formed (Figure 5C, ○) accounts for the ebselen selenoxide that disappeared ( ). The major part of the reaction was complete after incubation for 2 min (Figure 5C). After incubation for 4 min, about 95% of the ebselen selenoxide was reduced

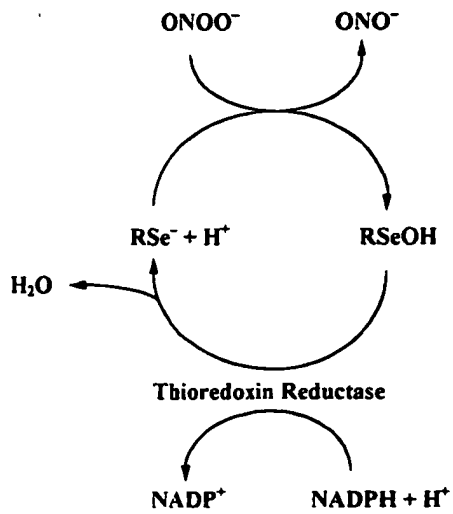

Figure 6. Proposed mechanism for thioredoxin reductase-mediated reduction of peroxynitrite to nitrite (or peroxynitrous acid to nitrous acid). The mechanism is based on that established for the reduction of ROOH to ROH by thioredoxin reductase and selenocysteine (*11*), as well as on previously reported peroxynitrite reductase activity for glutathione peroxidase and the mimic, ebselen (*6*, *7*). Initial reduction of the diselenide of selenocystine by thioredoxin reductase requires 2 equiv of NADPH.

to ebselen, suggesting a 1:1 stoichiometry with added NADPH.

Discussion

TR and Selenocystine. TR alone had no apparent peroxynitrite reductase activity under the conditions used. However, it was effective in the presence of selenocystine (Figure 1 and Table 1). Peroxynitrite-induced oxidation was significantly suppressed (Figure 1A), and nitrite formation increased (Figure 1B). These effects tended to follow the time course of NADPH oxidation (Figure 4), suggesting an enzymatic effect. Selenocystine is reduced by mammalian TR with an apparent K_m of 6 μM and a k_{cat} of 3200 min^{-1} (*9*), which is more than 4 orders of magnitude faster than the k_{cat} for reduction of selenocystine by GSH (*19*). Since TR and NADPH efficiently reduce selenocystine directly, Trx is most likely unnecessary for maintaining selenocysteine in the reduced state. This is in agreement with the observation here that Trx produced no significant enhancement of the peroxynitrite reductase activity of TR coupled with selenocystine and NADPH (Figure 1). Figure 6 schematically depicts the proposed mechanism of thioredoxin reductase-mediated reduction of peroxynitrite in the presence of selenocysteine. Peroxynitrite is reduced to nitrite by the selenol of selenocysteine; the resultant selenenic acid is then reduced by thioredoxin reductase at the expense of NADPH. While de novo synthesis of free selenocysteine reportedly does not occur in vivo, free selenocysteine is formed by the decomposition of selenoproteins or by the transsulfuration of selenomethionine (see ref *20* for review). Free selenocysteine is converted to selenide by selenocysteine β-lyase, but the enzyme is slow with a reported K_m of 0.83 mM (*21*). Therefore, low concentrations of selenocysteine may be present inside the cell and could be involved in defense against peroxynitrite.

TR and Ebselen. Results similar to those described above for selenocystine were observed with ebselen (Figures 2 and 4). Ebselen has been shown previously to have an affinity for TR, competitively inhibiting the Trx-dependent reduction of insulin by TR with an apparent K_i of 2.8 μM (*22*). It is shown here that TR and NADPH can efficiently reduce the selenoxide of ebselen in the absence of Trx (Figure 5). These data support the hypothesis that the reaction of ebselen with peroxynitrite can be catalytically maintained in the presence of a reducing system. The efficient reduction of ebselen selenoxide to ebselen by TR and NADPH suggests that this enzyme may be relevant in vivo. Figure 6 also depicts the proposed mechanism by which the reaction with ebselen and peroxynitrite is maintained catalytically by thioredoxin reductase at the expense of NADPH.

Role of Trx in the TR and Ebselen–Albumin Complex. Upon administration, extracellular ebselen is present in human plasma as an albumin complex (*23*), most likely as a selenenyl sulfide with the reactive cysteine residue on the protein (*24*). In the experiments described here, TR increased nitrite formation during peroxynitrite infusion in the presence of ebselen complexed with BSA (Figure 3). However, unlike selenocystine (Figure 1B) or free ebselen (Figure 2B), Trx enhanced nitrite formation under these conditions (Figure 3). These data suggest that while TR and NADPH, in the absence of Trx, can reduce the selenenyl sulfide complex of BSA–ebselen and release free ebselen, Trx increases the efficiency of the reaction; once free, ebselen should react as depicted in Figure 6, but it is possible that the reaction occurs also in the bound form. While the reported concentration of Trx in human plasma is quite low [~3 nM (*25*)], this does not take into account localized concentrations of Trx in blood or turnover of the protein by secretion from endothelial cells and blood cells, both of which have been shown to contain high levels of Trx and TR (*26*, *27*). Previous work has shown that while plasma cannot support the reduction by BSA–ebselen of cholesteryl linoleate hydroperoxides in low-density lipoprotein, whole blood can (*16*), supporting the hypothesis that there is a cellular process providing reducing equivalents in whole blood not found in plasma.

Concluding Remarks. The contribution of the mammalian thioredoxin system to antioxidant defense has previously been investigated. While the thioredoxin system has been shown to directly reduce lipid hydroperoxides (*11*, *28*), the reaction rate is relatively slow. However, the ability of the thioredoxin system to reduce more potent antioxidants may be biologically significant. For example, ascorbate (*29*), glutathione peroxidases (*12*), and thioredoxin peroxidases (*27*) are reduced by the thioredoxin system. The observations here not only support the hypothesis that the thioredoxin system may contribute to antioxidant defense but also suggest that it may play a specific role in peroxynitrite defense. The recent observation that L929 murine fibrosarcoma cells overexpressing Trx are resistant to the cytotoxicity of SIN-1 (*30*) supports this hypothesis.

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References

(1) Beckman, J. S. (1996) The physiological and pathophysiological chemistry of nitric oxide. In *Nitric oxide: principles and actions* (Lancaster, J., Ed.) pp 1–82, Academic Press, San Diego, CA.

(2) Briviba, K., Roussyn, I., Sharov, V. S., and Sies, H. (1996) Attenuation of oxidation and nitration reactions of peroxynitrite by selenomethionine, selenocystine and ebselen. *Biochem. J.* **319**, 13–15.

(3) Masumoto, H., and Sies, H. (1996) The reaction of ebselen with peroxynitrite. *Chem. Res. Toxicol.* **9**, 262–267.

(4) Salgo, M. G., Stone, K., Squadrito, G. L., Battista, J. R., and Pryor, W. A. (1995) Peroxynitrite causes DNA nicks in plasmid pBR322. *Biochem. Biophys. Res. Commun.* **210**, 1025–1030.

(5) Roussyn, I., Briviba, K., Masumoto, H., and Sies, H. (1996) Selenium-containing compounds protect DNA from single-strand breaks caused by peroxynitrite. *Arch. Biochem. Biophys.* **330**, 216–218.

(6) Sies, H., and Masumoto, H. (1997) Ebselen as a glutathione peroxidase mimic and as a scavenger of peroxynitrite. *Adv. Pharmacol.* **38**, 229–246.

(7) Sies, H., Sharov, V. S., Klotz, L.-O., and Briviba, K. (1997) Glutathione peroxidase protects against peroxynitrite-mediated oxidations. A new function for selenoproteins as peroxynitrite reductase. *J. Biol. Chem.* **272**, 27812–27817.

(8) Arteel, G. E., Mostert, V., Oubrahim, H., Briviba, K., Abel, J., and Sies, H. (1998) Protection by selenoprotein P in human plasma against peroxynitrite-mediated oxidation and nitration. *J. Biol. Chem.* **379**, 1201–1205.

(9) Björnstedt, M., Kumar, S., Björkhem, L., Spyou, G., and Holmgren, A. (1997) Selenium and the thioredoxin and glutaredoxin systems. *Biomed. Environ. Sci.* **10**, 271–279.

(10) Kumar, S., Björnstedt, M., and Holmgren, A. (1992) Selenite is a substrate for calf thymus thioredoxin reductase and thioredoxin elicits a large non-stoichiometric oxidation of NADPH in the presence of oxygen. *Eur. J. Biochem.* **207**, 435–439.

(11) Björnstedt, M., Hamberg, M., Kumar, S., Xue, J., and Holmgren, A. (1995) Human thioredoxin reductase directly reduces lipid hydroperoxides by NADPH and selenocystine strongly stimulates the reaction via catalytically generated selenols. *J. Biol. Chem.* **270**, 11761–11764.

(12) Björnstedt, M., Xue, J., Huang, W., Åkesson, B., and Holmgren, A. (1994) The thioredoxin and glutaredoxin systems are efficient electron donors to human plasma glutathione peroxidase. *J. Biol. Chem.* **269**, 29382–29384.

(13) Tamura, T., and Stadtman, T. C. (1996) A new selenoprotein from human lung adenocarcinoma cells: purification, properties, and thioredoxin reductase activity. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 1006–1011.

(14) Zhong, L., Arner, E. S. J., Ljung, J., Aslund, F., and Holmgren, A. (1998) Rat and calf thioredoxin reductase are homologous to glutathione reductase with a carboxyl-terminal elongation containing a conserved catalytically active penultimate selenocysteine residue. *J. Biol. Chem.* **273**, 8581–8591.

(15) Koppenol, W. H., Kissner, R., and Beckman, J. S. (1996) Syntheses of peroxynitrite: to go with the flow or on solid grounds? *Methods Enzymol.* **269**, 296–302.

(16) Christison, J., Sies, H., and Stocker, R. (1994) Human blood cells support the reduction of low-density-lipoprotein-associated cholesteryl ester hydroperoxides by albumin-bound ebselen. *Biochem. J.* **304**, 341–345.

(17) Szabó, C., Ferrer-Sueta, G., Zingarelli, B., Southan, G. J., Salzman, A. L., and Radi, R. (1997) Mercaptoethylguanidine and guanidine inhibitors of nitric-oxide synthase react with peroxynitrite and protect against peroxynitrite-induced oxidative damage. *J. Biol. Chem.* **272**, 9030–9036.

(18) Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991) Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.* **266**, 4244–4250.

(19) Yasuda, K., Watanabe, H., Yamazaki, S., and Toda, S. (1980) Glutathione peroxidase activity of D,L-selenocystine and selenocystamine. *Biochem. Biophys. Res. Commun.* **96**, 243–249.

(20) Burk, R. (1991) Molecular biology of selenium with implications for its metabolism. *FASEB J.* **5**, 2274–2279.

(21) Esaki, N., Nakamura, T., Tanaka, H., and Soda, K. (1982) Selenocysteine lyase, a novel enzyme that specifically acts on selenocysteine. *J. Biol. Chem.* **257**, 4386–4391.

(22) Engman, L., Cotgreave, I., Angulo, M., Taylor, C. W., Paine-Murrieta, G. D., and Powis, G. (1997) Diaryl chalcogenides as selective inhibitors of thioredoxin reductase and potential antitumor agents. *Anticancer Res.* **17**, 4599–4606.

(23) Wagner, G., Schuch, G., Akerboom, T. P. M., and Sies, H. (1994) Transport of ebselen in plasma and its transfer to binding sites in the hepatocyte. *Biochem. Pharmacol.* **48**, 1137–1144.

(24) Ullrich, V., Weber, P., Meisch, F., and von Appen, F. (1996) Ebselen-binding equilibria between plasma and target proteins. *Biochem. Pharmacol.* **52**, 15–19.

(25) Nakamura, H., Vaage, J., Valen, G., Padilla, C. A., Björnstedt, M., and Holmgren, A. (1998) Measurements of plasma glutaredoxin and thioredoxin in healthy volunteers and during open-heart surgery. *Free Radical Biol. Med.* **24**, 1176–1186.

(26) Rozell, B., Hansson, H.-A., Luthman, M., and Holmgren, A. (1985) Immunohistochemical localization of thioredoxin and thioredoxin reductase in adult rats. *Eur. J. Cell Biol.* **38**, 79–86.

(27) Cha, M.-K., and Kim, I.-H. (1995) Thioredoxin-linked peroxidase activity from human red blood cell: Evidence for the existence of thioredoxin and thioredoxin reductase in human red blood cell. *Biochem. Biophys. Res. Commun.* **217**, 900–907.

(28) Mitsui, A., Hirakawa, T., and Yodoi, J. (1992) Reactive oxygen-reducing and protein-refolding activities of adult T cell leukemia-derived factor/human thioredoxin. *Biochem. Biophys. Res. Commun.* **186**, 1220–1226.

(29) Mendiratta, S., Qu, Z.-C., and May, J. M. (1998) Enzyme-dependent ascorbate recycling in human erythrocytes: role of thioredoxin reductase. *Free Radical Biol. Med.* **25**, 221–228.

(30) Takagi, Y., Gon, Y., Todaka, T., Nozaki, K., Nishiyama, A., Sono, H., Hashimoto, N., Kikuchi, H., and Yodoi, J. (1998) Expression of thioredoxin is enhanced in atherosclerotic plaques and during neointima formation in rat arteries. *Lab. Invest.* **78**, 957–966.

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Human Thioredoxin Reductase Directly Reduces Lipid Hydroperoxides by NADPH and Selenocystine Strongly Stimulates the Reaction via Catalytically Generated Selenols*

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Human placenta thioredoxin reductase (HP-TR) in the presence of NADPH-catalyzed reduction of (15*S*)-hydroperoxy-(5*Z*),(8*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid ((15*S*)-HPETE) into the corresponding alcohol ((15*S*)-HETE). Incubation of 50 nM HP-TR and 0.5 mM NADPH with 300 μM 15-HPETE for 5 min resulted in formation of 16.5 μM 15-HETE. After 60 min, 74.7 μM 15-HPETE was reduced. The rate of the reduction of 15-HPETE by the HP-TR/NADPH peroxidase system was increased 8-fold by the presence of 2.5 μM selenocystine, a diselenide amino acid. In this case, 15-HPETE was catalytically reduced by the selenol amino acid, selenocysteine, generated from the diselenide by the HP-TR/NADPH system. To a smaller extent, selenodiglutathione or human thioredoxin also potentiated the reduction of 15-HPETE by HP-TR. Hydrogen peroxide and 15-HPETE were reduced at approximately the same rate by HP-TR, thioredoxin, and selenocystine. In contrast, *t*-butyl hydroperoxide was reduced at a 10-fold lower rate. Our data suggest two novel pathways for the reduction and detoxification of lipid hydroperoxides, hydrogen peroxide, and organic hydroperoxides, *i.e.* the human thioredoxin reductase-dependent pathway and a coupled reduction in the presence of selenols or selenide resulting from the reduction of selenocystine or selenodiglutathione.

Polyunsaturated fatty acids can be oxygenated into hydroperoxides, either non-enzymatically (1) or in the presence of specific lipoxygenases (2). Thus, arachidonic acid, an abundant polyunsaturated fatty acid in tissues, can provide (15*R,S*)-HPETE[1] and its regioisomers by non-enzymatic oxygenation as well as (15*S*)-HPETE when exposed to arachidonic acid 15-lipoxygenase (3). Further conversion of 15-HPETE, enzymatically and non-enzymatically, leads to the formation of epoxy alcohols and trihydroxyeicosatrienoic acids (4), to dioxygenated derivatives such as 5,15-diHPETE, 8,15-diHETE, and 14,15-diHETE (3) and to lipoxins (5). In addition, glutathione peroxidase-catalyzed reduction of 15-HPETE into the corresponding hydroxy acid (15-HETE) is likely to have importance for *in vivo* detoxification of 15-HPETE and other fatty acid hydroperoxides.

Thioredoxin (Trx) is a ubiquitous 12-kDa protein with the conserved active-site sequence -Cys-Gly-Pro-Cys- located on a protrusion in its three-dimensional structure (6). Oxidized thioredoxin with an active-site disulfide is reduced by NADPH and thioredoxin reductase (TR), and reduced thioredoxin is a powerful general protein disulfide reductase (6, 7). Since its original isolation from *Escherichia coli* as a hydrogen donor for the enzyme ribonucleotide reductase essential for the synthesis of deoxyribonucleotides and DNA (8), many new functions have been discovered (6, 7). Thus, thioredoxin can regulate the activity of enzymes, receptors, or transcription factors via thiol-redox control (7). Adult T-cell leukemia-derived factor is a secreted form of human thioredoxin operating as a cytokine (9). Thioredoxin reductase is a FAD-containing dimeric enzyme that has been purified and characterized from a variety of species (10). Mammalian TR has a broad substrate specificity and reacts not only with its homologous Trx but also with *E. coli* Trx (11), 5,5'-dithiobis(2-nitrobenzoic acid) (11), GS-Se-SG (12), selenite (13), vitamin K (14), alloxan (15), and the active-site selenocysteine residue in glutathione peroxidases (GSH-Px) (16).

Selenium is an essential trace element that is known to have antioxidant properties. The most established mechanism behind the antioxidant function is the formation of selenocysteine from selenide and the incorporation of this amino acid in the active site of glutathione peroxidases (17). These enzymes detoxify hydrogen peroxide, lipid hydroperoxides, and organic hydroperoxides (18). Selenite and GS-Se-SG are both efficiently reduced directly by mammalian TR or the complete thioredoxin system (12, 13). In these reactions selenide (HSe^-) is formed and recycles with oxygen giving rise to a non-stoichiometric large oxidation of NADPH. Recently we showed that thioredoxin reductase with or without reduced thioredoxin is an electron donor to human plasma GSH-Px in the reduction of hydroperoxides (16).

The aim of the present study was to investigate whether thioredoxin reductase and thioredoxin can reduce lipid hydroperoxides and if low molecular weight selenium compounds could act as charge transfer catalysts. This could be an important alternative pathway for the detoxification of hydroperoxides in addition to GSH-Px-mediated reduction.

EXPERIMENTAL PROCEDURES

(15*S*)-HPETE (purity, in excess of 96%) was obtained by incubation of arachidonic acid (Nu-Chek-Prep, Inc., Elysian, MN) with soybean lipoxygenase at 0 °C (19). (15*R,S*)-HPETE was prepared by autoxidation of arachidonic acid followed by isolation by silicic acid chromatography and straight-phase HPLC (*cf.* Ref. 20). (15*S*)-HETE was prepared in quantitative yield by reduction of (15*S*)-HPETE with triphenylphos-

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[1] The abbreviations used are: HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; TR, thioredoxin reductase; Trx, thioredoxin; HP-TR, human placenta thioredoxin reductase; CT-TR, calf thymus thioredoxin reductase; HPLC, high performance liquid chromatography; GS-Se-SG, selenodiglutathione; GR, glutathione reductase; GSH-Px, glutathione peroxidase.

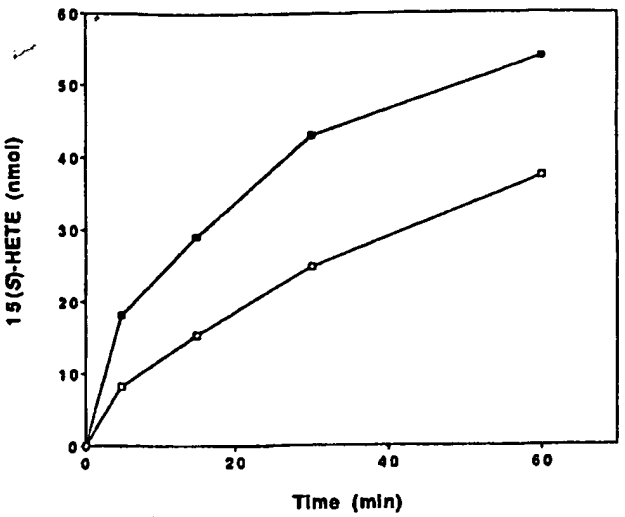

FIG. 1. **Time course of reduction of (15*S*)-HPETE in the presence of TR and NADPH.** (15*S*)-HPETE (300 μM) was added to 0.5 ml of 50 mM Tris-HCl, 1 mM EDTA, pH 7.5, containing NADPH (0.5 mM) and either 50 nM) HP-TR (□) or 50 nM CT-TR (■). The mixtures were kept at room temperature, and at the times indicated, the reactions were terminated by freezing in dry ice/ethanol. The hydroperoxide and its corresponding alcohol were extracted with diethyl ether and analyzed by straight-phase HPLC as described in the text.

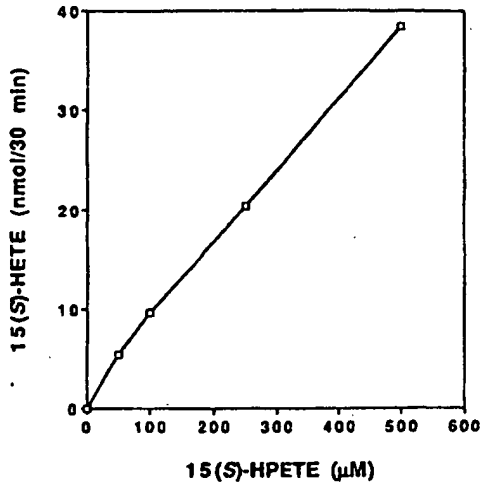

FIG. 2. **Reduction of (15*S*)-HPETE as a function of substrate concentration.** (15*S*)-HPETE (0–500 μM) was added to 0.5 ml of HP-TR (50 nM), NADPH (0.5 mM), and EDTA (1 mM) in 50 mM Tris-HCl buffer, pH 7.5, and kept at room temperature for 30 min. The reaction mixtures were extracted with diethyl ether and analyzed by straight-phase HPLC.

phine in diethyl ether. *t*-Butyl hydroperoxide and yeast GR were from Sigma. DL-Selenocystine was from Serva. TR from human placenta and calf thymus was purified essentially as described by Luthman and Holmgren (14). Enzyme activities were standardized by using 5,5′-dithiobis(2-nitrobenzoic acid) as a substrate (14). *E. coli* TR was a homogenous preparation as described previously (13). Recombinant human Trx was prepared as described by Ren *et al.* (21) and was reduced prior to use with dithiothreitol followed by desalting over a Sephadex G-25 column. GS-Se-SG was prepared as described by Björnstedt *et al.* (12).

Spectrophotometric Measurements—The reduction of selenocystine was performed in 50 mM Tris-HCl, 1 mM EDTA, pH 7.5, with 200 μM NADPH. The reduction was followed at 340 nm as oxidation of NADPH using a mM extinction coefficient of 6.2. To both the reference and the sample cuvette was added HP-TR. The reactions were started by addition of selenocystine to the sample cuvette and an equal volume of buffer to the reference cuvette. The final volume was 0.50 ml.

Spectrophotometric Determination of Peroxidase Activity—The reactions were performed in 50 mM Tris-HCl, 1 mM EDTA, pH 7.5, with 500 μM NADPH. The reduction of hydroperoxides was followed as oxidation of NADPH at 340 nm. To the cuvettes were added HP-TR, Trx, and selenocystine to final concentrations of 50 nM, 2.0 μM, and 2.5 μM, respectively. The reactions were started by addition of peroxide (150 μM) to the sample cuvette and an equal volume of 99.5% ethanol to the reference cuvette.

Determination of Peroxidase Activity by HPLC Analysis of (15S)-HPETE and (15S)-HETE—To a reaction mixture composed of 50 mM Tris-HCl, 1 mM EDTA, pH 7.5, and 0.5 mM or 1 mM NADPH was added either TR, TR + Trx, TR + selenocystine, TR + GS-Se-SG, or GR. The reactions were started by addition of (15*S*)-HPETE to a final concentration of 300 μM. After incubation at room temperature for 15 min (if not otherwise indicated) the reactions were terminated by rapid freezing in dry ice/ethanol. Incubation mixtures of 15-HPETE (0.5 ml) were acidified to pH 4 and rapidly extracted with 7 ml of diethyl ether. The ether phase was washed three times with water and taken to dryness. The residue was analyzed by straight-phase HPLC using a column of Nucleosil 50–5 (250 × 4.6 mm; Macherey-Nagel (Düren, Germany)) and 2-propanol/hexane/acetic acid (1.5:98.5:0.02, v/v/v) at a flow rate of 1.5 ml/min. The absorbance at 235 nm of the effluent was monitored and the digitized signal integrated using a Macintosh SE/30. The retention volumes of (15*S*)-HETE and (15*S*)-HPETE were 10.2–10.9 and 12.8–13.5 ml, respectively.

RESULTS

Reduction of Lipid Hydroperoxides by Mammalian Thioredoxin Reductase—(15*S*)-HPETE (300 μM) incubated with HP-TR or CT-TR and NADPH was reduced to (15*S*)-HETE (Fig. 1). In order to investigate possible stereospecificity, (15*R*,*S*)-HPETE was incubated with CT-TR/NADPH and the 15-HETE produced (32% reduction) as well as the remaining 15-HPETE (68%) were subjected to steric analysis (22). Both compounds were found to be virtually racemic (ratio of *S* to *R* enantiomers, 49.9:50.1 (15-HETE) and 50.8:49.2 (15-HPETE)). Thus there was no discrimination between enantiomers in the CT-TR-promoted reduction of 15-HPETE. The initial part of the reaction was faster using CT-TR compared with HP-TR (Fig. 1). There was an almost linear relation between the concentration of (15*S*)-HPETE and product formed up to 500 μM concentration of (15*S*)-HPETE (Fig. 2). Addition of Trx to HP-TR increased the rate of the reduction of (15*S*)-HPETE by 60% (Fig. 3) indicating that both proteins of the human Trx system can reduce lipid hydroperoxides. In contrast, neither *E. coli* thioredoxin reductase nor yeast glutathione reductase had any significant peroxidase activity (Fig. 3).

Reduction of Selenocystine by Mammalian Thioredoxin Reductase—Addition of HP-TR (50 nM) to selenocystine resulted in a very rapid oxidation of a stoichiometric amount of NADPH consistent with cleavage of each molecule of selenocystine into two molecules of selenocysteine (Fig. 4). Addition of GS-Se-SG or selenite to mammalian TR results in redox cycling with oxygen and a large non-stoichiometric oxidation of NADPH (12, 13). In the reaction between TR and selenocystine, especially at high concentrations of selenocystine, the oxidation of NADPH continued after oxidation of a stoichiometric amount (Fig. 4). This is consistent with redox cycling with oxygen. Thus, selenocysteine is autoxidized by oxygen, and the resulting selenocystine is again reduced by TR.

Reduction of Lipid Hydroperoxides by Catalytically Regenerated Selenols—The presence of selenocystine (2.5 μM) increased the peroxidase activity of HP-TR 8-fold (Fig. 3). After 15 min of incubation of HP-TR and selenocystine 72.5% of (15*S*)-HPETE (or 108.8 nmol) was reduced. Similar results were obtained with two other fatty acid hydroperoxides, *i.e.* the α-linolenic acid-derived (13*S*)-hydroperoxy-(9*Z*),(11*E*),(15*Z*)-octadecatrienoic acid and (9*S*)-hydroperoxy-(10*E*),(12*Z*),(15*Z*)-octadecatrienoic acid (data not shown). The presence of Trx only marginally increased the rate of the selenocysteine-coupled reaction (Fig. 3). Even when used in 0.5 μM concentration, selenocystine

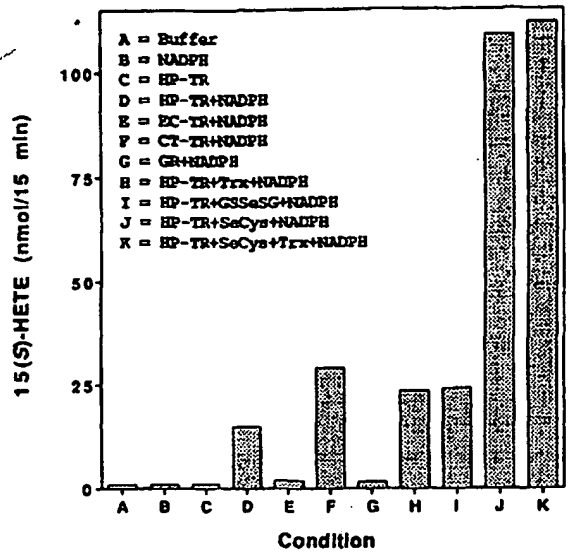

FIG. 3. **Reduction of (15*S*)-HPETE under various conditions.** (15*S*)-HPETE (300 μM) was added to the agents indicated dissolved in 0.5 ml of 50 mM Tris-HCl, 1 mM EDTA, pH 7.5. The mixtures were kept at room temperature for 15 min. *A*, buffer control; *B*, 1 mM NADPH; *C*, 50 nM HP-TR; *D*, 50 nM HP-TR plus 1 mM NADPH; *E*, 50 nM *E. coli* thioredoxin reductase (*EC-TR*) plus 1 mM NADPH; *F*, 50 nM CT-TR plus 1 mM NADPH; *G*, 1 unit of yeast GR plus 1 mM NADPH; *H*, 50 nM HP-TR, 2 μM Trx, and 1 mM NADPH; *I*, 50 nM HP-TR, 5 μM GS-Se-SG, and 1 mM NADPH; *J*, 50 nM HP-TR, 2.5 μM selenocystine, and 1 mM NADPH; *K*, 50 nM HP-TR, 2.5 μM selenocystine, 2 μM Trx, and 1 mM NADPH. The reaction mixtures were extracted with diethyl ether and analyzed by straight-phase HPLC. The values are averages of at least two individual experiments.

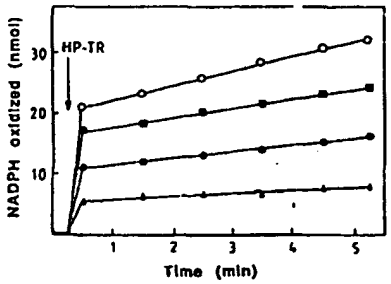

FIG. 4. **Reduction of selenocystine by TR.** The reactions were performed in 50 mM Tris-HCl, 1 mM EDTA, pH 7.5, with 200 μM NADPH. To both the reference and the sample cuvette was added selenocystine to final concentrations of 10 μM (▲), 20 μM (●), 30 μM (■), and 40 μM (○). The reactions were started by addition of TR to the sample cuvette and an equal volume of buffer to the reference cuvette (final volume, 500 μl). The reactions were followed at 340 nm.

approximately doubled the rate of the TR/Trx-dependent peroxidase reaction (data not shown). Hydrogen peroxide and (15*S*)-HPETE were reduced at approximately the same rate by HP-TR, Trx, and selenocystine (Fig. 5). However, the rate of reduction of *t*-butyl hydroperoxide was slower. Addition of GS-Se-SG (5 μM) to HP-TR increased the rate of the reaction by 60% (Fig. 3). GS-Se-SG will react with TR and form selenide (12). The results show that selenide can act as a charge transfer catalyst between TR and hydroperoxides. As a control, selenocystine (2.5 μM) without TR and NADPH was added to (15*S*)-HPETE (300 μM). The extent of reduction (0.6%) of the hydroperoxide did not differ from that observed with buffer (Fig. 3).

DISCUSSION

Our results show that lipid hydroperoxides can be reduced by TR. The mechanism of TR-dependent peroxidase activity is

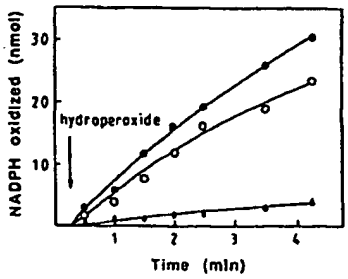

FIG. 5. **Reduction of different hydroperoxides.** The reactions were performed in 50 mM Tris-HCl, 1 mM EDTA, pH 7.5, with 500 μM NADPH. To the reference as well as the sample cuvette were added HP-TR (50 nM), Trx (2 μM), and selenocystine (2.5 μM). The reactions were started by addition of *t*-butyl hydroperoxide (▲), (15*S*)-HPETE (○), or H_2O_2 (●) to the sample cuvette in a final concentration of 150 μM and an equal volume of buffer or ethanol to the reference cuvette (final volume, 500 μl). The reduction of hydroperoxide was followed as oxidation of NADPH at 340 nm.

likely as shown in Fig. 6*A*. Trx has previously been shown to slowly reduce hydrogen peroxide and other non-disulfide reagents if coupled with NADPH and thioredoxin reductase (14, 15, 23, 24).

Selenocystine is a diselenide amino acid (25, 26). Upon reduction selenocystine is decomposed into two molecules of the selenol amino acid selenocysteine (26). This amino acid is an essential component of several proteins including GSH-Px (17). In mammalian cells selenocysteine is incorporated in proteins by a selenocysteinyl-$tRNA^{Ser}$ that recognizes the nonsense codon UGA (27). Free selenocysteine is produced in mammalian tissues by decomposition of selenoproteins and trans-sulfuration of selenomethionine (28). Selenocysteine is catabolized by selenocysteine β-lyase into L-alanine and selenide (29). However, the K_m of this enzyme is high (0.83 mM) (29), indicating that it is important only at rather high concentrations of selenocysteine. In the present study it was shown that selenocystine was efficiently reduced by TR and NADPH. Experiments with low concentrations of TR indicated that the kinetic parameters for the reaction with selenocystine are similar to that of the natural substrate thioredoxin, *i.e.* a low K_m and a high turnover number.[2] The very fast reduction of selenocystine by TR suggests that selenocystine is kept reduced intracellularly.

Rotruck *et al.* (30) in 1973 reported the presence of selenium as a component of GSH-Px. The field of selenium and antioxidant functions have expanded to include intracellular and extracellular GSH-Px (28) as well as synthetic selenium compounds like ebselen (31). GSH has been considered to be the only electron source for selenium-dependent hydroperoxide reduction, but human plasma contains essentially no free GSH (32). Recently we showed that the human Trx system is an efficient electron donor to human plasma GSH-Px (16). In this reaction TR or TR + Trx regenerates the charge of the active-site selenocysteine, which is oxidized during the peroxidase reaction. To investigate if this selenium-coupled peroxidase activity of TR was dependent on selenium inserted in a protein, *i.e.* GSH-Px, we added free selenocystine and GS-Se-SG to TR. The selenium-coupled reaction involves regeneration of Cys-Se^- from Cys-SeOH (Fig. 6*B*), in accordance with the regeneration of the active-site charge of GSH-Px (16). Cys-Se^- reduces hydroperoxides to the corresponding alcohol. At high concentrations of selenocystine, reduction of Cys-SeOH by Cys-Se^- and formation of Cys-Se-Se-Cys will occur. A possible mechanism for the potentiation of TR-dependent peroxidase activity by

[2] M. Björnstedt, M. Hamberg, and A. Holmgren, manuscript in preparation.

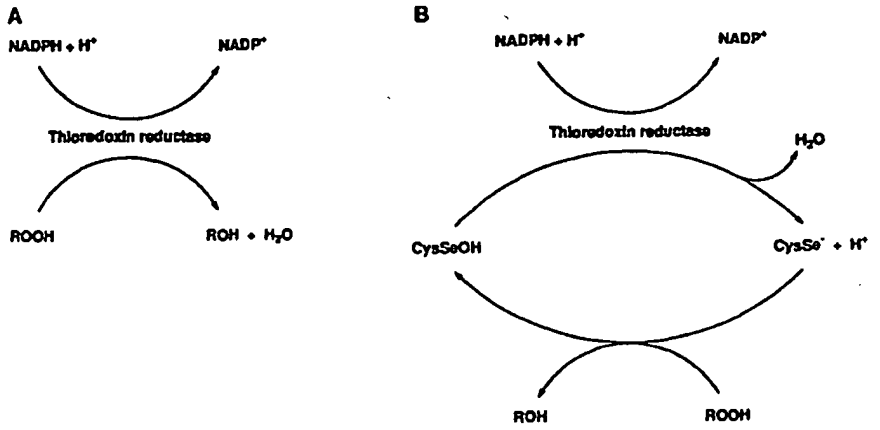

FIG. 6. **Proposed mechanism for the TR-dependent reduction of hydroperoxides.** *A*, TR peroxidase reaction; *B*, selenolate (Cys-Se$^-$)-coupled peroxidase reaction.

GS-Se-SG is the regeneration of HSe^- from HSeOH. One explanation for the higher activity of selenocystine compared with selenide is that the nucleophilicity of the selenium atom in selenocysteine should be greater than that of selenium in selenide.

Selenocystine was previously reported to have a low GSH-Px activity together with 2 mM GSH and GR (25). However, this activity is unlikely to be of physiological significance since the pH optimum for the reaction was 8.0 and the reaction was observed with 10.0 μM selenocystine and the maximum turnover based on the V_{max} was only 0.01 × min^{-1} (25). In fact sodium selenite was found to be more active than selenocystine. In the present study we found that the turnover of the selenol with hydroperoxide in the reaction catalyzed by TR was 6 × min^{-1}.

Unsaturated fatty acid hydroperoxides serve as intermediates in the formation of biologically important compounds such as leukotrienes and lipoxins. In other situations, lipid hydroperoxides can accumulate in tissues and exert harmful effects. Specifically, 15-HPETE, the hydroperoxide used in the present study, can oxidatively modify low density lipoprotein and has been implicated in atherosclerosis (33). One pathway for the elimination of lipid hydroperoxides involves reduction in the presence of glutathione peroxidase and glutathione. The present work gives evidence for another mechanism for detoxification of lipid hydroperoxides, *i.e.* the reduction into alcohols in the presence of thioredoxin reductase and NADPH. This pathway and its high capacity when operating together with catalytic amounts of selenocysteine could serve as an important alternative to glutathione peroxidase.

REFERENCES

1. Gardner, H. W. (1989) *Free Radical Biol. & Med.* 7, 65–66
2. Yamamoto, S. (1992) *Biochim. Biophys. Acta* 1128, 117–131
3. Ford-Hutchinson, A. W. (1991) *Eicosanoids* 4, 65–74
4. Bryant, R. W., and Bailey, J. M. (1981) *Prog. Lipid Res.* 20, 279–281
5. Serhan, C. N., Hamberg, M., and Samuelsson, B. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 5335–5339
6. Holmgren, A. (1985) *Annu. Rev. Biochem.* 54, 237–271
7. Holmgren, A. (1989) *J. Biol. Chem.* 264, 13963–13966
8. Thelander, L., and Reichard, P. (1979) *Annu. Rev. Biochem.* 48, 133–158
9. Yodoi, J., and Tursz, T. (1991) *Adv. Cancer Res.* 57, 381–411
10. Williams, C. H. J. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., ed) Vol. 3, pp. 121–211, CRC Press, Inc., Boca Raton, FL
11. Holmgren, A. (1977) *J. Biol. Chem.* 252, 4600–4606
12. Björnstedt, M., Kumar, S., and Holmgren, A. (1992) *J. Biol. Chem.* 267, 8030–8034
13. Kumar, S., Björnstedt, M., and Holmgren, A. (1992) *Eur. J. Biochem.* 207, 435–439
14. Luthman, M., and Holmgren, A. (1982) *Biochemistry* 21, 6628–6633
15. Holmgren, A., and Lyckeborg, C. (1980) *Proc. Natl. Acad. Sci. U. S. A.* 77, 5149–5152
16. Björnstedt, M., Xue, J., Huang, W., Åkesson, B., and Holmgren, A. (1994) *J. Biol. Chem.* 269, 29382–29384
17. Böck, A., Forchhammer, K., Heider, J., Leinfelder, W., Sawers, G., Veprek, B., and Zinoni, F. (1991) *Mol. Microbiol.* 5, 515–520
18. Flohé, L. (1989) in *GSH: Chemical, Biochemical and Medical Aspects* (Dolphin, B., Poulson, R., and Avramovich, O., eds) Part A, pp. 644–729, John Wiley & Sons, Inc., New York
19. Hamberg, M., and Samuelsson, B. (1967) *J. Biol. Chem.* 242, 5329–5335
20. Hamberg, M. (1989) *Lipids* 24, 249–255
21. Ren, X., Björnstedt, M., Shen, B., Ericson, M. L., and Holmgren, A. (1993) *Biochemistry* 32, 9701–9708
22. Zhang, L.-Y., and Hamberg, M. (1994) *Chem. Phys. Lipids* 74, 151–161
23. Mitsui, A., Hirakawa, T., and Yodoi, J. (1992) *Biochem. Biophys. Res. Commun.* 186, 1220–1226
24. Holmgren, A. (1979) *J. Biol. Chem.* 254, 9113–9119
25. Yasuda, K., Watanabe, H., Yamazaki, S., and Toda, S. (1980) *Biochem. Biophys. Res. Commun.* 96, 243–249
26. Tanaka, H., and Soda, K. (1987) *Methods Enzymol.* 143, 240–244
27. Lee, B. J., Worland, J. N., Davis, J. N., Stadtman, T. C., and Hatfield, D. (1989) *J. Biol. Chem.* 264, 9724–9727
28. Burk, R. F. (1991) *FASEB J.* 5, 2274–2279
29. Esaki, N., Nakamura, T., Tanaka, H., and Soda, K. (1982) *J. Biol. Chem.* 257, 4386–4391
30. Rotruck, J. T., Pope, A. L., Ganther, H. E., Swanson, A. B., Hafeman, D. G., and Hoekstra, W. G. (1973) *Science* 179, 588–590
31. Maiorino, M., Roveri, A., Coassin, M., and Ursini, F. (1988) *Biochem. Pharmacol.* 37, 2267–2271
32. Wendel, A., and Cikryt, P. (1980) *FEBS Lett.* 120, 209–211
33. Ylä-Herttuala, S., Rosenfield, M. E., Parthasarathy, S., Glass, C. K., Sigal, E., Witztum, J. L., and Steinberg, D. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 6959–6963

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Selenite is a substrate for calf thymus thioredoxin reductase and thioredoxin and elicits a large non-stoichiometric oxidation of NADPH in the presence of oxygen

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The thioredoxin system, comprising NADPH, thioredoxin reductase and thioredoxin reduces protein disulfides via redox-active dithiols. We have discovered that sodium selenite is a substrate for the thioredoxin system; 10 μM selenite plus 0.05 μM calf thymus thioredoxin reductase at pH 7.5 caused a non-stoichiometric oxidation of NADPH (100 μM after 30 min). In contrast, thioredoxin reductase from *Escherichia coli* showed no direct reaction with selenite, but addition of 3 μM *E. coli* thioredoxin also resulted in non-stoichiometric oxidation of NADPH, consistent with oxidation of the two active-site thiol groups in thioredoxin to a disulfide. Kinetically, the reaction was complex with a lag phase at low selenite concentrations. Under anaerobic conditions the reaction stopped after 1 mol selenite had oxidized 3 mol NADPH; the admission of air then resulted in continued consumption of NADPH consistent with autooxidation of selenium intermediate(s). Ferricytochrome *c* was effectively reduced by calf thymus thioredoxin reductase and selenite in the presence of oxygen. Selenite caused a strong dose-dependent inhibition of the formation of thiol groups from insulin disulfides with either the *E. coli* or calf-thymus thioredoxin system. Thus, under aerobic conditions selenite catalyzed, NADPH-dependent redox cycling with oxygen, a large oxygen-dependent consumption of NADPH and oxidation of reduced thioredoxin inhibiting its disulfide-reductase activity.

Selenium is now known to be an essential trace element for a wide range of living organisms, including man [1–3]. Some forms of selenium show potent anti-carcinogenic and anti-neoplastic activity [4–10]. Inorganic selenium compounds, like sodium selenite, strongly stimulate cell growth at approximately 50 nM and inhibits at greater than 1 μM [11]. This biphasic effect on cell growth in tissue culture occurs by unknown mechanisms [11]. The most established function of selenium in mammalian cells is as an essential selenocysteine residue in the active site of glutathione peroxidase, a detoxifying enzyme [12]. However, changes in the function of glutathione peroxidase do not seem to be directly involved in the effects of selenite on cell growth [11, 13].

It has long been known that selenite reacts with thiols [14, 15]. We have therefore investigated if selenium compounds interact with the thioredoxin system (thioredoxin, NADPH and thioredoxin reductase) which is a hydrogen donor for ribonucleotide reductase [16–18]. The thioredoxin system is also a multifunctional disulfide-reducing system present in all species including *Escherichia coli* and mammalian cells [18]. Thioredoxin reductase catalyzes the reduction of oxidized thioredoxin ($Trx\text{-}S_2$) by NADPH, and reduced thioredoxin [$Trx\text{-}(SH)_2$] is the disulfide reductase (Eqns 1–3):

$$Trx\text{-}S_2 + NADPH + H^+ \xrightarrow{\text{Trx-S}_2\text{ reductase}} Trx(SH)_2 + NADP^+ \quad (1)$$

$$Trx\text{-}(SH)_2 + \text{protein-}S_2 \rightarrow Trx\text{-}S_2 + \text{protein-}(SH)_2. \quad (2)$$

$$\text{Net: } NADPH + H^+ + \text{protein-}S_2 \rightarrow NADP^+ + \text{protein-}(SH)_2. \quad (3)$$

We have discovered that selenite serves as an excellent substrate for a mammalian thioredoxin system. The nature of these reactions provide new mechanisms for oxidation of NADPH, thioredoxin reductase and thioredoxin of importance for the effects of selenite on cellular functions and growth.

MATERIALS AND METHODS

Dithiothreitol, Na_2SeO_3, Na_2SeO_4, NADPH, superoxide dismutase, 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2), ferricytochrome *c* and catalase were from Sigma. Bovine insulin was from Nordisk Insulin A/S, Gentofte, Denmark.

Enzyme preparations

Thioredoxin from *E. coli* [19] and calf thymus [20] were homogenous preparations. Thioredoxin reductase from *E. coli* was purified to homogeneity from A237/pPMR14, an *E. coli* strain containing the cloned *trxB* gene [21]. This was generously provided by Dr. Marjorie Russel, Rockefeller University, New York. Thioredoxin reductase from calf thymus was

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Abbreviations. $Trx\text{-}S_2$, oxidized thioredoxin, $Trx\text{-}(SH)_2$, reduced thioredoxin, GS-Se-SG, selenodiglutathione; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

Enzymes. Thioredoxin reductase (EC 1.6.4.5); superoxide dismutase (EC 1.15.1.1); catalase (EC 1.11.1.6).

urified to homogeneity essentially as described for rat liver [22] except that the enzyme was eluted from ADP-Sepharose by a gradient of 0.01 – 0.50 M potassium phosphate, pH 7.5, and a final chromatography step on Superose 12 was used. The enzyme (M_r 116000) was stored at 8 mg/ml in 50 mM Tris/Cl, 1 mM EDTA, pH 7.5, at −70°C. Dilutions of the enzyme for the experiments were made to 1.0 mg/ml (8.6 μM) in the same buffer, kept in an ice bath and were frozen at −20°C between experiments, without loss of activity.

Spectrophotometric measurements

The activity with selenite was determined in semimicro quartz cuvettes at room temperature using a Zeiss PMQ3 spectrophotometer equipped with an automatic sample exchanger and a recorder. Measurements were done either in air or anaerobically in carefully evacuated and argon-equilibrated special cuvettes. The reaction was started by injecting the enzyme through a rubber septum with a gas-tight syringe.

Enzyme assays

Measurements of selenite reduction were performed in 50 mM Tris/Cl, 1 mM EDTA, pH 7.5, generally with 200 μM NADPH. Control cuvettes, used as reference cells contained either no enzyme or no selenite and were automatically subtracted by the spectrophotometer. The oxidation of NADPH was followed at 340 nm using an $\varepsilon_{340} = 6.2\ mM^{-1} \cdot cm^{-1}$.

Assays of the disulfide-reducing activity of the thioredoxin system were based on reduction of insulin as described previously [22]. Incubations in a final volume of 120 μl contained 80 mM Hepes, pH 7.6, 3 mM EDTA, 0.7 mM NADPH, 330 μM insulin and the indicated amounts of thioredoxin, selenite and thioredoxin reductase. After incubation for 20 min at 37°C (in air) the reaction was stopped by 0.50 ml 6 M guanidine/HCl, 0.20 M Tris/Cl, pH 8.0, 0.2 mg/ml Nbs_2 and the absorbance at 412 nm was measured to determine SH groups ($\varepsilon_{412} = 13.6\ mM^{-1} \cdot cm^{-1}$). Controls contained no thioredoxin or no thioredoxin reductase.

Reduction of ferricytochrome *c*

Reduction of ferricytochrome *c* was followed at 550 nm with a Zeiss PMQ3 spectrophotometer using the method described by Bromberg and Pick [23]. The reaction was performed in semimicro quartz cuvettes at room temperature in 500 μl 100 μM ferricytochrome *c* in 65 mM potassium phosphate, pH 7.0, 170 mM sucrose, 2 mM sodium azide, 1 mM EGTA, 10 μM FAD with 50 nM calf thymus thioredoxin reductase and selenite. The change in A_{550} following addition of selenite was used to quantify the reduction of ferricytochrome *c* using $\varepsilon_{550} = 2.1 \times 10^4\ M^{-1} \cdot cm^{-1}$. Superoxide dismutase (30 U) and catalase (40 μg) were included in the assay to check the involvement of superoxide radicals. To obtain anaerobic conditions, the cuvettes were carefully evacuated of air and flushed with argon. Selenite solutions evacuated of air and flushed with argon were used to start the reactions.

RESULTS

Reactions with calf thymus thioredoxin reductase

During studies of the effect of Na_2SeO_3 on the disulfide-reducing activity of the thioredoxin system, we noted oxidation of NADPH in cuvettes containing only selenite and calf thymus thioredoxin reductase. With 0.05 μM enzyme an extensive oxidation of NADPH was observed at A_{340} (Fig. 1). No oxidation of NADPH by selenite was recorded in the absence of the enzyme. Low concentrations of selenite (1 – 10 μM) gave a pronounced lag phase to the reaction. The oxidation of NADPH was non-stoichiometric and 5 nmol selenite caused the oxidation of 60 nmol NADPH in 60 min (Fig. 1).

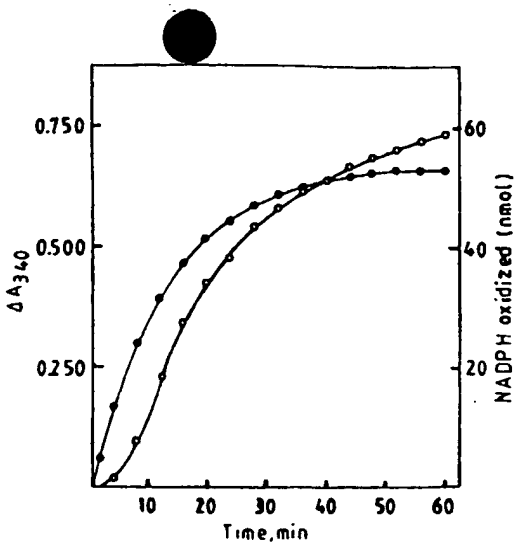


Fig. 1. Selenite-dependent oxidation of NADPH catalyzed by calf thymus thioredoxin reductase. Two cuvettes contained 0.50 ml 50 mM Tris/Cl, 2 mM EDTA, pH 7.5, 200 μM NADPH and 50 nM calf thymus thioredoxin reductase. Addition of 10 μM (○) or 40 μM (●) sodium selenite to the sample cuvette resulted in oxidation of NADPH recorded at 25°C (in air).

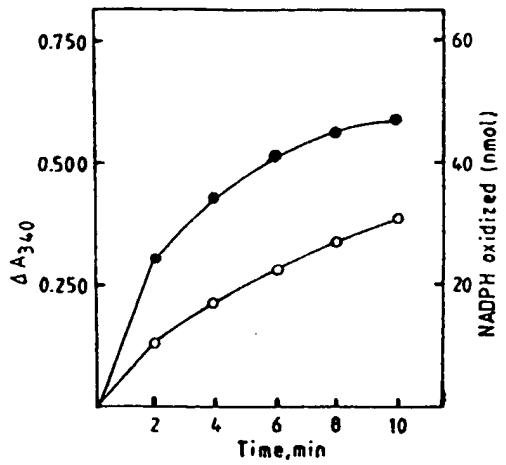


Fig. 2. Effect of enzyme concentration on NADPH oxidation with 230 μM selenite. 50 nM (○) or 100 nM (●) calf thymus thioredoxin reductase was incubated with 200 μM NADPH and selenite in 50 mM Tris/Cl, 1 mM EDTA, pH 7.5.

The effect of a high (230 μM) concentration of selenite and varying concentrations of thioredoxin reductase is illustrated in Fig. 2. The reaction was proportional to the amount of enzyme (at 2 min) but the rate with both concentrations decreased with time. The reaction of NADPH with selenite was also catalyzed by rat liver thioredoxin reductase with the same efficiency as the calf thymus enzyme (data not shown).

Use of sodium selenate in place of selenite with calf thymus thioredoxin reductase failed to give any oxidation of NADPH.

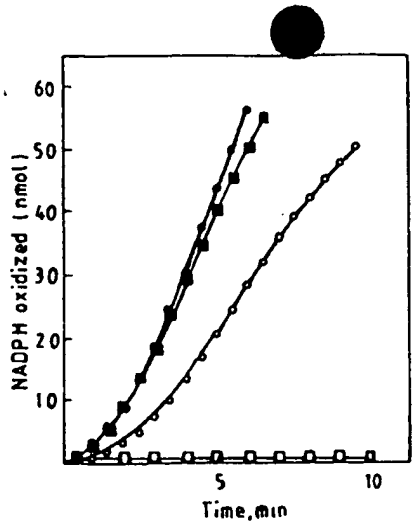


Fig. 3. Selenite-dependent oxidation of NADPH catalyzed by *E. coli* and calf thymus thioredoxin reductase in the presence or absence of thioredoxin. The oxidation of NADPH was recorded in the presence of 10 μM selenite in 0.50 ml 50 mM Tris/Cl, 2 mM EDTA, pH 7.5, containing 200 μM NADPH with (□) 100 nM *E. coli* thioredoxin reductase or (■) 100 nM thioredoxin reductase plus 3 μM *E. coli* thioredoxin and (○) 100 nM calf thymus thioredoxin reductase or (●) 100 nM calf thymus thioredoxin reductase plus 3 μM calf thymus thioredoxin.

Reactions with *E. coli* thioredoxin reductase and thioredoxin

Thioredoxin reductase from *E. coli* was also tested for its reactivity with 10 μM selenite. As shown in Fig. 3, no NADPH oxidation was observed and selenite is thus not a substrate for this enzyme. However, after addition of 3 μM oxidised thioredoxin a fast reaction was observed (Fig. 3). Thus, also reduced *E. coli* thioredoxin catalyzes reduction of selenite.

Effect of calf thymus thioredoxin

Thioredoxin from *E. coli* and calf thymus have an identical active-site sequence (Cys-Gly-Pro-Cys) and show the same activity in protein-disulfide reductions [18, 24]. The effect of the calf thymus thioredoxin on the reduction of 10 μM selenite was tested by using 100 nM calf thymus thioredoxin reductase with or without 3 μM thioredoxin (Fig. 3). A clear stimulation of the activity was seen in the presence of thioredoxin and selenite is thus reduced by both the calf thymus thioredoxin reductase and thioredoxin.

Determination of the relationship between reaction rate and substrate concentration

Determination of the K_m for selenite in the presence of air with the thioredoxin system was complicated by the shape of the progress curves with a lag phase at low selenite concentrations (Fig. 1). This phenomenon was independent of buffer (Tris/Cl or phosphate), the presence or absence of albumin (0.2 mg/ml) or EDTA (1 – 10 mM). We, therefore, chose to plot the maximal rate of NADPH oxidation against increasing substrate concentrations. The concentration of selenite giving half-maximal velocity was 5 μM for the *E. coli* system (Fig. 4A) and 20 μM for calf thymus thioredoxin reductase (Fig. 4B).

Reduction of selenite under anaerobic conditions

Since a non-stoichiometric amount of NADPH was oxidized by selenite in the presence of the thioredoxin system

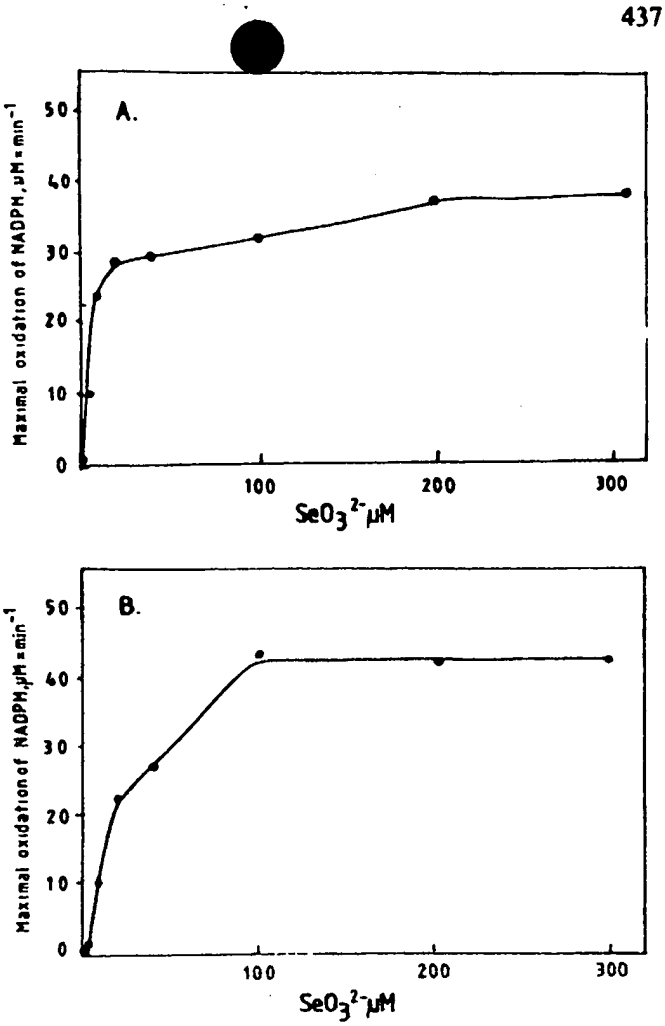


Fig. 4. Effect of varying selenite concentrations on NADPH oxidation catalyzed by the *E. coli* thioredoxin system or calf thymus thioredoxin reductase. (A) Activity was determined in 0.1 M potassium phosphate, 10 mM EDTA, pH 7.0, 250 μM NADPH and 0.20 mg/ml bovine serum albumin with 50 nM *E. coli* thioredoxin reductase and 3 μM *E. coli* Trx. (B) Activity was determined in 50 mM Tris/Cl, 1 mM EDTA, pH 7.5, 250 μM NADPH with 100 nM calf thymus thioredoxin reductase. The maximal rate of NADPH oxidation is shown.

under ordinary aerobic conditions (Figs 1 and 3), we investigated the oxygen dependence of the reaction. Therefore, experiments were performed under anaerobic conditions. As seen from Fig. 5, anaerobic reduction of selenite with calf thymus thioredoxin reductase stopped after oxidation of 3 mol NADPH/mol selenite. The initial rate with aerobic and anaerobic conditions were identical (data not shown). Admission of air resulted in a continued reaction, consistent with reoxidation of a product. The result suggests the following overall stoichiometry of the reaction (Eqn 4):

$$SeO_3^{2-} + 3\,NADPH + 3\,H^+ \xrightarrow{\text{calf thymus Trx-S}_2\text{ reductase}} Se^{2-} + 3\,NADP^+ + 3\,H_2O. \quad (4)$$

Thus, anaerobically selenide (Se^{2-}) should be generated. This is known to be highly sensitive to auto-oxidation [6].

Reduction of ferricytochrome *c* with calf thymus thioredoxin reductase in the presence of selenite

Calf thymus thioredoxin reductase (50 nM) catalyzed aerobically a non-stoichiometric reduction of ferricytochrome

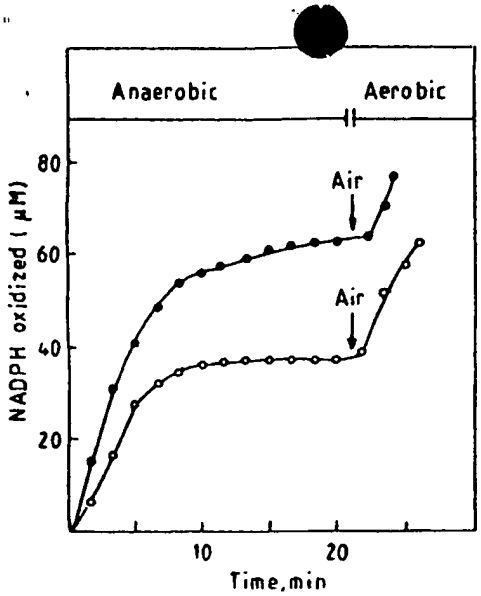


Fig. 5. Oxidation of NADPH by calf thymus thioredoxin reductase and selenite under anaerobic and aerobic conditions. Reaction mixtures consisted of 100 mM Tris/Cl, 2 mM EDTA, pH 7.5, 200 μM NADPH and the indicated concentrations of selenite. Anaerobic conditions were obtained by evacuation of air from reaction mixture and equilibration with oxygen-free argon in special cuvettes. The reaction was started by injecting 50 nM calf thymus thioredoxin reductase through a rubber septum. Aerobic conditions were created by admission of air and mixing. Controls contained the enzyme but no selenite.

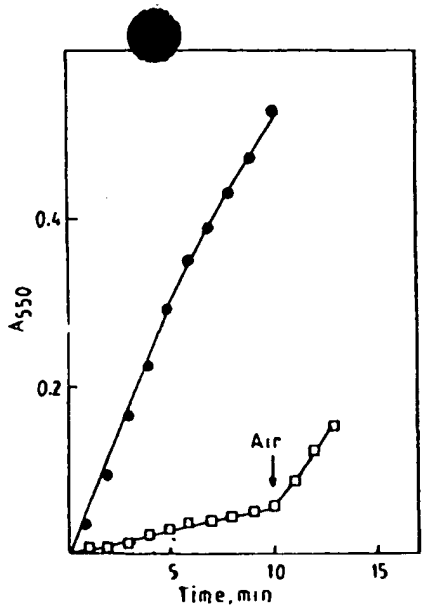


Fig. 6. Aerobic/anaerobic reduction of ferricytochrome *c* by calf thymus thioredoxin reductase with selenium as an electron-transfer catalyst. Calf thymus thioredoxin reductase (50 nM) was incubated with 200 μM NADPH, 100 μM ferricytochrome *c*, 170 mM sucrose, 2 mM sodium azide, 1 mM EGTA, 10 μM FAD in 65 mM potassium phosphate, pH 7, at room temperature with or without 40 μg catalase and 15 U superoxide dismutase; (●) aerobically or (□) anaerobically. The change in A_{550} was recorded after addition of selenite (20 μM) to the sample cuvette.

c with 20 μM selenite (Fig. 6). To investigate the oxygen dependence, the reaction was also studied anaerobically. The rate of cytochrome-*c* reduction was dramatically decreased by removal of air from the system. Under anaerobic conditions, there was a slow reaction. Inclusion of superoxide dismutase (15 U) and catalase (40 μg) in the assay mixture did not influence the rate of aerobic ferricytochrome-*c* reduction excluding the involvement of superoxide radicals or H_2O_2.

Effects of selenite on insulin-disulfide reduction by the thioredoxin system

Selenite inhibited insulin-disulfide reduction by the thioredoxin system (Eqns 1 – 3) as shown in Table 1. Reactions involving calf thymus thioredoxin reductase, which also is active with *E. coli* thioredoxin [25] were particularly sensitive to inhibition. This is consistent with selenite reacting both directly with the enzyme and also with reduced thioredoxin, thereby blocking insulin-disulfide reduction. As seen from Table 1, an almost total inhibition of insulin reduction was seen with higher concentration of selenite and 50% inhibition was observed below 10 μM selenite in all combinations, demonstrating a powerful effect on protein-disulfide reduction.

Table 1. Effect of sodium selenite on insulin-disulfide reduction catalyzed by the thioredoxin system. All assays contained 80 mM Hepes buffer, pH 7.6, 3 mM EDTA and 0.7 mM NADPH along with the indicated final concentrations of thioredoxin reductase, thioredoxin and sodium selenite in a volume of 120 μl. Following incubation at 37°C for 20 min the reaction was stopped with 0.5 ml 6 M guanidine/HCl, 0.2 M Tris/Cl, pH 8.0, 0.2 mg Nbs_2/ml and SH-group concentrations were determined at 412 nm. Activity is given as μM SH groups formed in 120 μl. Reaction conditions were (A) 7 nM *E. coli* thioredoxin reductase with 18 μM *E. coli* thioredoxin; (B) 10 nM calf thymus thioredoxin reductase with 18 μM *E. coli* thioredoxin; (C) 10 nM calf thymus thioredoxin reductase with 3 μM calf thymus thioredoxin; (D) 100 nM calf thymus thioredoxin reductase with 0.3 μM calf thymus thioredoxin.

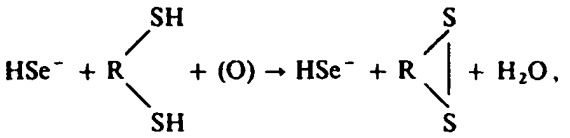
Sodium selenite concentration	Disulfide reduction under condition			
	A	B	C	D
μM	μM SH group generated/20 min			
–	420	330	583	300
10	146	76	76	100
40	75	38	52	100
200	26	7	17	54
400	26	9	10	12

DISCUSSION

Sodium selenite was a good substrate for calf thymus thioredoxin reductase and thioredoxin reductase plus thioredoxin from *E. coli*, causing oxygen-dependent and non-stoichiometric oxidation of NADPH. Addition of calf thymus thioredoxin to the mammalian reductase resulted in increased reaction rate, showing that selenite and reactive intermediates were substrates for both proteins of the mammalian thioredoxin system. We have recently found that selenodiglutathione (GS-Se-SG) is a substrate for calf thymus thioredoxin reductase, causing continous aerobic oxidation of NADPH [26]. Furthermore, GS-Se-SG is a very efficient oxidant of reduced *E. coli* thioredoxin [26].

After addition of selenite, the reaction showed a pronounced lag phase, especially at low concentrations. This lag phase is consistent with the formation of an active metabolite responsible for the non-stoichiometric oxygen-dependent oxidation of NADPH. There is no lag phase in the reaction between mammalian thioredoxin reductase and GS-Se-SG; instead there is a fast stoichiometric oxidation of NADPH followed by a slower continued reaction [26]. Ferricytochrome

c was effectively reduced by mammalian thioredoxin reductase and selenite. This reduction was fast in the presence of oxygen but slow under anaerobic conditions. Selenium radicals formed in the reaction between selenite and thiols are known to reduce cytochrome *c* [27]. The highly efficient oxygen-dependent reduction of ferricytochrome *c* by selenite and the thioredoxin system suggests the existence and recycling of a selenium intermediate in oxidation-state −II, such as HSe^- or H_2Se. Ganther [14] suggested that H_2Se, formed in the reaction between GS-Se-SG and glutathione reductase, reacts with oxygen and the result is formation and precipitation of elemental selenium which leads to an end of the reaction. This is not likely to occur in the reaction between selenium compounds and the thioredoxin system where the non-stoichiometric reaction probably is explained by the recycling of a reactive intermediate. This intermediate will act as a charge-transfer catalyst transferring hydrogen atoms from NADPH via the thioredoxin system to oxygen. The stoichiometry of the oxygen-dependent reaction is thus likely to be as suggested for the oxygen-dependent reaction between GS-Se-SG and the thioredoxin system [26]:



where R is the active site of thioredoxin or mammalian thioredoxin reductase. This suggested reaction is also supported by our results that superoxide dismutase and catalase do not influence the reduction of ferricytochrome *c* by selenite and thioredoxin reductase. These data exclude the involvement of superoxide radicals and H_2O_2. Anaerobically the stoichiometry was found to be 3 mol oxidized NADPH/mol selenite, indicating the formation of selenide (Se^{2-}).

When fully reduced ribonuclease A was treated with molar equivalents of selenite, SH groups were oxidized and the products were elemental selenium and inactive protein molecules cross-linked by so-called selenodisulfide bridges (-S-Se-S-) [28]. In view of these results, we initially expected inhibition of the activity of thioredoxin or thioredoxin reductase by selenite since these proteins contain two redox-active cysteine residues in their active sites [18, 29]. The mechanism of thioredoxin reductase involves transport of electrons via FAD to the redox-active disulfide to form a dithiol. The reaction with selenite probably involves consecutive two-electron reductions and oxidation of the dithiol to a disulfide with no stable selenodisulfide intermediate.

Thioredoxin reductase from mammalian cells is known to have a broad substrate specificity and react not only with its homologous thioredoxin but also with *E. coli* thioredoxin [25], Nbs_2 [25], vitamin K [22] and alloxan [30]. In this respect, the activity with selenite is in line with the broader substrate specificity, a property not found for *E. coli* thioredoxin reductase. The difference between thioredoxin reductase from *E. coli* and calf thymus is also reflected in the size; both have two identical subunits but the subunit of the mammalian enzyme has a M_r 58000 compared to that of *E. coli*, M_r 35000.

The lack of reactivity of the *E. coli* thioredoxin reductase with selenite permitted direct demonstration of the reaction between selenite and reduced *E. coli* thioredoxin; also reduced calf thymus thioredoxin reacted with selenite.

Recently Tagaya et al. [31] found human thioredoxin to be expressed and secreted as a growth factor by activated lymphocytes. Furthermore, thioredoxin has been suggested to have important roles in the growth process of virally infected lymphocytes [32]. In the mammalian system, selenite will oxidize both thioredoxin and thioredoxin reductase and thus act as both a substrate and a competitive inhibitor of mammalian thioredoxin reductase, inhibiting its reduction of thioredoxin. Changes in the redox status of the thioredoxin system by selenite might be of great importance via the physiological functions of this system. A possible cytotoxic mechanism of pharmacological doses of selenite could be oxidative stress due to fast NADPH oxidation resulting from interactions between the thioredoxin system and selenite.

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REFERENCES

1. Schwartz, K. & Foltz, C. M. (1957) *J. Am. Chem. Soc. 79*, 3292–3293.
2. Stadtman, T. C. (1974) *Science 183*, 915–922.
3. Spallholz, J. E., Martin, J. L. & Ganther, H. F. (1981) *Selenium in biology and medicine*, AVI Publishing Co., Westport, Connecticut.
4. Vernie, L. N. (1984) *Biochim. Biophys. Acta 738*, 203–217.
5. Shamberger, R. J. (1983) *Biochemistry of selenium*, Plenum Press, New York.
6. Milner, J. A. (1985) *Fed. Proc. 44*, 2568–2572.
7. Ip, C. (1985) *Fed. Proc. 44*, 2573–2578.
8. Medina, D., Lane, H. W. & Tracey, C. M. (1983) *Cancer Res. 43*, 2460–2464.
9. Le Boef, R. A., Laishes, B. A. & Hockstra, W. G. (1985) *Cancer Res. 45*, 5496–5504.
10. Greeder, G. A. & Milner, J. A. (1980) *Science 209*, 825–827.
11. Medina, D. & Oborn, C. J. (1984) *Cancer Res. 44*, 4361–4365.
12. Stadtman, T. C. (1980) *Annu. Rev. Biochem. 49*, 93–110.
13. Lane, H. W. & Medina, D. (1983) *Cancer Res. 43*, 1558–1561.
14. Ganther, H. E. (1971) *Biochemistry 10*, 4089–4098.
15. Tsen, C. C. & Tappel, A. L. (1958) *J. Biol. Chem. 233*, 1230–1232.
16. Thelander, L. & Reichard, P. (1979) *Annu. Rev. Biochem. 48*, 133–158.
17. Holmgren, A. (1989) *J. Biol. Chem. 264*, 13963–13966.
18. Holmgren, A. (1985) *Annu. Rev. Biochem. 54*, 237–271.
19. Holmgren, A. & Reichard, P. (1967) *Eur. J. Biochem. 2*, 187–196.
20. Engström, N. E., Holmgren, A., Larsson, A. & Söderhäll, S. (1974) *J. Biol. Chem. 249*, 205–210.
21. Russel, M. & Model, P. (1985) *J. Bacteriol. 163*, 238–242.
22. Luthman, M. & Holmgren, A. (1982) *Biochemistry 21*, 6628–6633.
23. Bromberg, Y. & Pick, E. (1985) *J. Biol. Chem. 260*, 13539–13545.
24. Holmgren, A. (1979) *J. Biol. Chem. 254*, 9627–9632.
25. Holmgren, A. (1977) *J. Biol. Chem. 252*, 4600–4606.
26. Björnstedt, M., Kumar, S. & Holmgren, A. (1992) *J. Biol. Chem., 267*, 8030–8034.
27. Levander, O. A., Morris, V. C. & Higgs, D. J. (1973) *Biochemistry 23*, 4591–4595.
28. Ganther, H. E. & Corcoran, C. (1969) *Biochemistry 8*, 2557–2563.
29. Holmgren, A. (1980) *Experentia (Basel) 36*, 149–180.
30. Holmgren, A. & Lyckeborg, C. (1980) *Proc. Natl Acad. Sci. USA 77*, 5149–5152.
31. Tagaya, Y., Maeda, Y., Mitsui, A., Kondo, H., Hamuro, J., Brown, N., Arai, K.-I., Yokata, T., Wakasugi, H. & Yodoi, J. (1989) *EMBO J. 8*, 757–764.
32. Wakasugi, N., Tagaya, Y., Wakasugi, H., Mitsui, A., Maeda, M., Yodoi, J. & Tursz, T. (1990) *Proc. Natl Acad. Sci. USA 87*, 8282–8286.

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A NOVEL BIOLOGICALLY ACTIVE SELENO-ORGANIC COMPOUND—I

GLUTATHIONE PEROXIDASE-LIKE ACTIVITY *IN VITRO* AND ANTIOXIDANT CAPACITY OF PZ 51 (EBSELEN)

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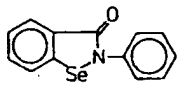
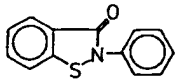
Abstract—A synthetic seleno-organic compound, 2-phenyl-1,2-benzoisoselenazol-3(2*H*)-one (PZ 51), exhibits GSH peroxidase-like activity *in vitro*, in contrast to its sulfur analog, PZ 25. In addition, PZ 51 behaves as an antioxidant shown by a temporary protection of rat liver microsomes against ascorbate/ADP-Fe-induced lipid peroxidation, an effect also elicited by PZ 25 but to a smaller extent. This protection against lipid peroxidation is independent of GSH and of P-450 monooxygenase activity.

Much of the role of selenium in biology can be attributed to the selenoenzyme glutathione peroxidase (GSH Px) [1–3]. Present as selenocysteinyl residue, the trace element undergoes a catalytic redox cycle involving the selenol and selenenic and/or seleninic acids [4, 5]. Since GSH Px catalyses the reduction of a wide variety of hydroperoxides, it together with GSH constitutes a powerful cellular defence system against so-called oxidative stress. As enzyme proteins may not readily reach intracellular target sites [6], the therapeutic use of GSH peroxidase as a tetrameric enzyme could be limited in systemic application, quite apart from potential immunological problems.

The synthesis of seleno-organic compounds which exhibit GSH Px-like activity has led to a series of substances [7], of which we have studied compound PZ51 and its sulfur analog, PZ25, as a reference compound (Scheme I); PZ25 is almost devoid of



PZ 51



PZ 25

Scheme I

GSH Px activity. The present report provides information on GSH Px-like activity *in vitro* and on an antioxidant capacity of these compounds in model systems which is independent of GSH. The latter was studied in the well-characterized microsomal system of lipid peroxidation initiated by ADP-Fe/ascorbate [8]; lipid peroxidation was assessed by the thiobarbituric acid-reactive material test, alkane production, and low-level chemiluminescence.

MATERIALS AND METHODS

Biological materials and chemicals. Liver microsomes were prepared from male Wistar rats fed on stock diet (Altromin, Lage, F.R.G.) and pretreated with phenobarbital (0.1% in the drinking water for 1 week). PZ 51 and PZ25 were kindly provided by A. Nattermann & Co. GmbH (Cologne, F.R.G.). Lyophilized GSH peroxidase (1 U/mg) from bovine erythrocytes was a gift from Prof. A. Wendel. Other chemicals and biochemicals were purchased from Fluka (Buchs, Switzerland), Merck (Darmstadt, F.R.G.), and Boehringer (Mannheim, F.R.G.).

Glutathione peroxidase activity. The assay mixture (700 μl) consisted of 50 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 1 mM GSH, 1 mM sodium azide, 0.25 mM NADPH, and 1 U/ml GSSG reductase. Absorbance at 366 nm was recorded, and then a sample containing GSH Px activity (usually 50 μl) was added to estimate the sample blank. Subsequently, hydroperoxide (H_2O_2, *t*-butyl hydroperoxide, or cumene hydroperoxide) was added; appropriate blanks were run in the absence of added GSH Px activity and in the presence of hydroperoxide. With no GSH in the assay mixture, there was no absorbance decrease with PZ 51 or PZ 25 in the concentration range studied.

Microsomal incubation. Microsomes were prepared as described in [9] and were stored at −18° before use. Incubations of microsomal fractions (1 mg protein/ml) were carried out under constant oxygenation at 37° in 7.5 ml of 0.1 M potassium phosphate buffer, pH 7.4, containing 2 mM ADP/16 μM $FeSO_4$. Lipid peroxidation was started upon addition of 0.5 mM ascorbate to the above incubation mixture.

Lipid peroxidation assays. The extent of lipid peroxidation in the above incubation mixture was assessed by the thiobarbituric acid-reactive material test (malondialdehyde accumulation) [10], alkane production [11], and low-level chemiluminescence [12, 13].

Malondialdehyde accumulation was measured at 535–570 nm ($\Delta\varepsilon = 156$/mM per cm) with a Sigma ZWS II Spectrophotometer (Biochem. Co., Munich, F.R.G.). Alkane evolution experiments were carried out in 43 ml-sealed flasks as described previously [14]. The system was calibrated with calibration gas (Messer-Griesheim, Duisberg, F.R.G.) and the amounts of alkanes were calculated using a correction formula for alkane dilution [11] and expressed in pmoles alkane formed per mg protein. Low-level chemiluminescence was measured with an EMI-9658 AM photomultiplier (EMI-Gencom, Plainview, New York, U.S.A.) sensitive in the 350–800 nm range. Assay conditions for chemiluminescence were described previously [14]. Results shown in the figures and the table are representative of three different microsome preparations.

RESULTS

Glutathione peroxidase activity of PZ 51 in vitro

The selenocompound PZ 51 exhibits a much more pronounced *t*-butylhydroperoxide-dependent GSH Px activity than the sulphur analog, PZ 25 (Fig. 1A). Correcting for the blank with only the hydroperoxide present, the GSH Px-like activity of the latter is almost zero. As shown in Fig. 1B, there is a linear dependence on the concentration of PZ 51 until about 20 μM, both with H_2O_2 and *t*-butyl hydroperoxide, and also with cumene hydroperoxide (not shown). The dependence of GSH Px-like activity of PZ51 and GSH Px on the concentration of GSH is shown in Fig. 1C. Whereas PZ51 shows saturation of its peroxidase-like activity beyond 2 mM GSH, GSH Px exhibits a linear response, indicating different kinetic behaviour.

Antioxidant activity of PZ 51 in microsomal lipid peroxidation

The experimental model used to study the effect of PZ 51 and PZ 25 on non-enzymic microsomal lipid peroxidation has been previously described [14]. This model consists in assessing the duration of the induction period or lag phase (τ_0), which normally precedes the initiation of the ascorbate/ADP-Fe-induced microsomal membrane lipid peroxidation.

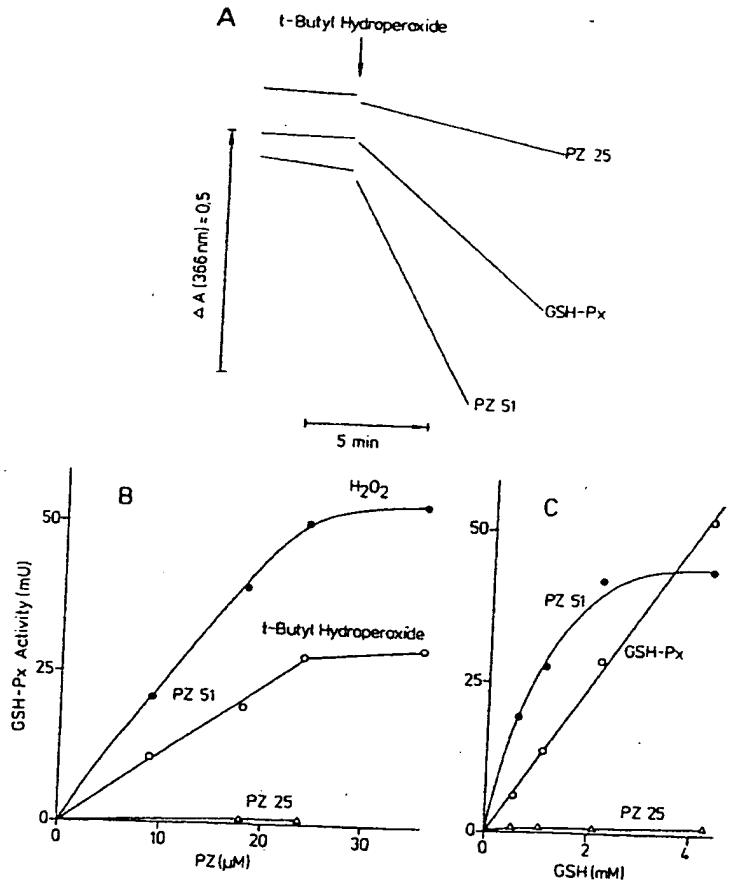


Fig. 1. GSH Peroxidase-like activity of PZ 51. (A) Absorbance at 366 nm in the assay carried out as described in Materials and Methods: note the higher blank with PZ 51 compared to PZ 25 (both 36 μM). The reaction was started upon addition of 1 mM *t*-butyl hydroperoxide. (B) Dependence of GSH peroxidase-like activity of PZ 51 and PZ 25 on concentration of the PZ compound assayed in the presence of 0.5 mM H_2O_2 or 1 mM *t*-butyl hydroperoxide, and 1 mM GSH. (C) Dependence of GSH peroxidase activity and GSH peroxidase-like activity of PZ 51 and PZ 25 on GSH concentration; measurements were carried out in the presence of 1 mM *t*-butyl hydroperoxide.

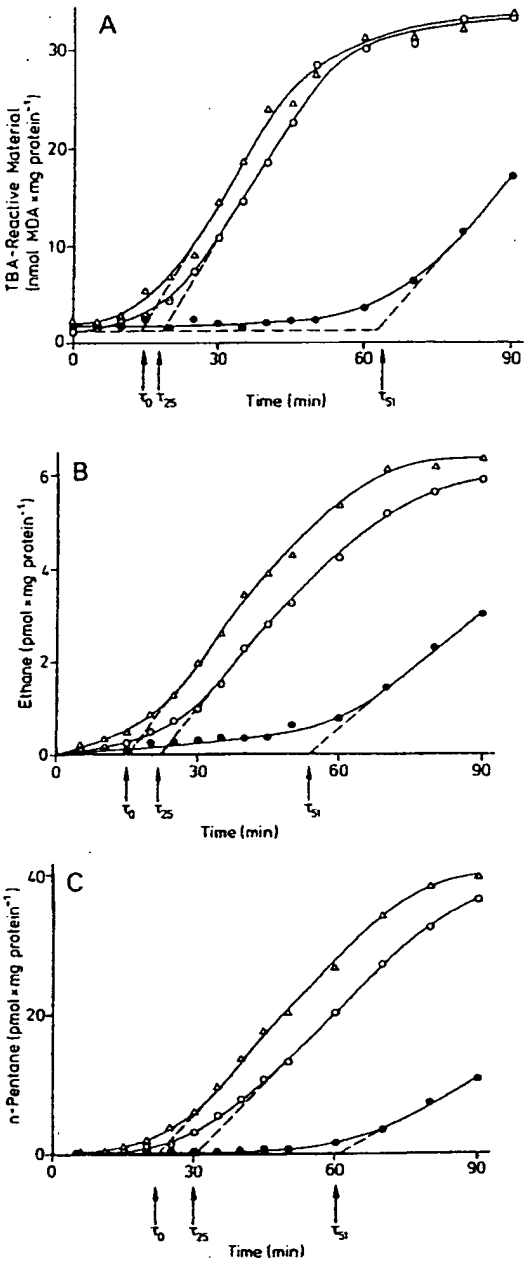


Fig. 2. Effect of PZ 51 and PZ 25 on ascorbate/ADP-Fe induced MDA (A), ethane (B) and *n*-pentane (C) production of microsomal fractions. Assay conditions as described in the Materials and Methods section and in Table 1. Time courses in the absence (△) and in the presence of PZ 51 (●) and PZ 25 (○). τ_0, induction period for control; τ_{51} for PZ 51 and τ_{25} for PZ 25.

τ_0 reflects, therefore, the occurrence of endogenous protective mechanisms against free radical attack to membrane lipids. This lag phase can be prolonged by antioxidants (τ_x).

PZ 51 (1.6 μM) considerably prolongs the lag phase (τ_{51}) of ascorbate/ADP-Fe-induced lipid peroxidation of rat liver microsomes as measured by malondialdehyde formation (Fig. 2A), alkane production (Figs. 2B and 2C), and low-level chemiluminescence (Fig. 3). A less pronounced effect is obtained with 1.6 μM PZ 25 (τ_{25}), as shown in the same figures. In spite of this prolongation of the induction period, the maximal malondialdehyde and alkane production, and chemiluminescence intensity are similar to control values. The difference between the induction periods in malondialdehyde and alkane (Fig. 2) on the one hand and chemiluminescence (Fig. 3) on the other hand might be due to the slightly different incubation procedures applied. The τ_0 and τ (1.6 μM PZ) values are listed in Table 1 for the parameters of lipid peroxidation. Since *n*-pentane has a greater lipid solubility than ethane, a slightly higher τ_0 value for *n*-pentane was observed.

The τ/τ_0 ratio, which indicates the relative increase in duration of the lag phase in the presence of PZ, would reflect the resistance of microsomes to lipid peroxidation [14]. Figure 4 shows the dependence of the τ/τ_0 ratio on PZ concentration for chemiluminescence measurements and emphasizes the greater efficiency of PZ 51 as compared to its sulphur analog PZ 25. The effect of PZ is dependent on microsomal protein concentration in the assay system (not shown), as already found with diethyldithiocarbamate in the same concentration range [14], and it might involve a specific binding to microsomal components. PZ 51 is also more effective than PZ 25 in preventing the onset of microsomal lipid peroxidation as reflected by alkane evolution (Fig. 5). At 60 min incubation time, ethane, *n*-butane, and *n*-pentane have been measured in the presence of different concentrations of PZ in the incubation mixture. Twofold higher amounts of PZ 25 than PZ 51 are necessary to observe the similar inhibitory effects on alkane production.

It should be mentioned that these effects of PZ

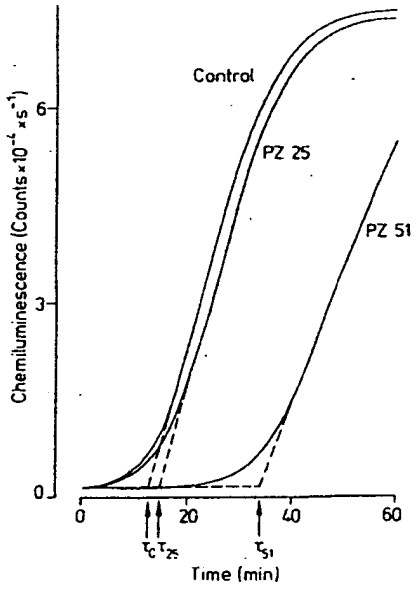


Fig. 3. Time course of chemiluminescence reaction in the absence and presence of PZ 51 and PZ 25. τ_0, τ_{51} and τ_{25} corresponding induction periods. Assay conditions as in Fig. 2.

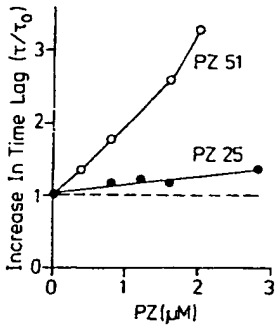


Fig. 4. Effect of different PZ 25 and PZ 51 concentrations on τ/τ_0 ratio estimated by chemiluminescence. Assay conditions as in Fig. 2.

Table 1. Induction periods of ascorbate/ADP-Fe induced lipid peroxidation from controls (τ_0), PZ 51 (τ_{51}) and PZ 25 (τ_{25}) determined by MDA, ethane and *n*-pentane production and chemiluminescence (CL) formation

Induction period (min)	MDA	Ethane	*n*-Pentane	CL
τ_0	14	15	22	12
τ_{51}	62	54	61	34
τ_{25}	18	22	30	15
τ_{51}/τ_0	4.4	3.6	2.8	2.8
τ_{25}/τ_0	1.3	1.5	1.4	1.3

Assay conditions as described in Materials and Methods. Controls: 10 μl DMSO. PZ 51 and PZ 25: 1.6 μM in 10 μl DMSO.

on microsomal fractions have been obtained in the absence of GSH in the incubation medium. This was necessary because of the antioxidant capacity of GSH itself [14–16], especially in the concentration range necessary to support the GSH Px-like activity of PZ 51.

The absence of reducing equivalents for the cytochrome P-450 monooxygenase system (as NADPH), as in the present non-enzymatic-induced lipid peroxidation, excludes a possible microsomal-dependent metabolism of the selenocompound, which might divert electrons to other pathways than the lipid peroxidation free radical process.

DISCUSSION

This study presents a synthetic seleno-organic compound, PZ 51, exhibiting a GSH Px-like activity *in vitro*; previous work by Caldwell and Tappel [17, 18] and Yasuda *et al.* [19] were centered on selenium-containing amino acid analogs (see Ref. 20 for a more detailed analysis). In addition, this new compound might display an antioxidant activity through a hypothetical free radical quenching capacity. However, it must be stressed that the term antioxidant is used in the present context as the capacity of retarding the initiation of lipid peroxidation, and it does not imply necessarily a free radical quenching activity.

The sulfur analog, PZ 25, which has no GSH Px-like activity, exerts an antioxidant property only in a higher concentration range. The molecular mechanism of this effect is not clear yet. Because the only difference between the two compounds is the exchange of Se for S, it may be assumed that the different efficiency of these compounds would be closely related to the selenium of sulphur moiety. The hydrophobic nature of these compounds might assure a better interaction with membrane lipids, thus accounting for the low concentration necessary to achieve this protecting effect against lipid peroxidation.

Whether PZ 51 is able to replace GSH Px in rats fed a selenium deficient diet, where GSH Px activity is decreased [17], or to provide an additional peroxidase activity against oxidative stress in rats fed a standard diet remains to be studied. Furthermore, it is necessary to investigate whether the effect of PZ 51 *in vitro* relies more on a peroxidase-like activity or on a more unspecific free radical quenching capacity (cf. Ref. 21).

Acknowledgements—We thank Ursula Rabe and Maria Zimmer for excellent technical assistance. This work was supported by Deutsche Forschungsgemeinschaft Schwerpunkt "Mechanismen toxischer Wirkungen von Fremdstoffen".

We thank Dr. E. Graf of Nattermann & Cie. GmbH, Cologne, for a generous gift of PZ 51 and PZ 25.

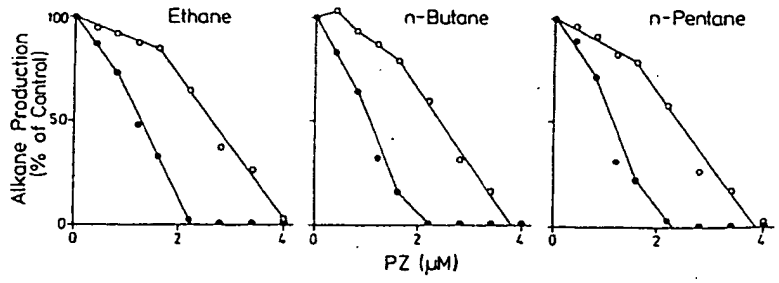


Fig. 5. Effect of PZ 25 (○) and PZ 51 (●) concentration on alkane production. Assay conditions as in Materials and Methods. PZ was dissolved in 10 μl DMSO, controls were with 10 μl DMSO. Control values for alkane production expressed in pmol × mg protein^{-1} in the PZ 25 experiment: Ethane, 6.0; *n*-butane, 1.5; *n*-pentane, 39.8; in the PZ 51 experiment: Ethane, 8.5; *n*-butane, 1.8; *n*-pentane, 51.4. Incubation time 60 min.

REFERENCES

1. G. C. Mills, *J. biol. Chem.* **234**, 502 (1957).
2. J. T. Rotruck, A. L. Pope, H. E. Ganther, A. B. Swanson, D. Hafeman and W. G. Hoekstra, *Science* **179**, 588 (1973).
3. L. Flohè, W. A. Günzler and H. H. Schock, *FEBS Lett.* **32**, 132 (1973).
4. A. Wendel, W. Pilz, R. Ladenstein, G. Sawatzki and U. Weser, *Biochim. biophys. Acta* **377**, 211 (1975).
5. H. E. Ganther, *Chemica Scripta* **8A**, 79 (1975).
6. A. M. Michelson, O. Dangeon, K. Puget, P. Durosay, B. Perdereau and C. Carbaroux, *Molec. Physiol.* **3**, 27 (1983).
7. Patents: Ger. Offen. 3027073/4/5, 3041036; Ger. Pat. Appl. P. 3226284/6; 3239387; A. Nattermann & Cie GmbH.
8. S. D. Aust and B. A. Svingen, in *Free Radicals in Biology*, Vol. V (Ed. W. A. Pryor), pp. 1–28. Academic Press, New York (1982).
9. R. F. Poyer and P. B. McCay, *J. biol. Chem.* **246**, 263 (1972).
10. F. Bernheim, M. L. C. Bernheim and K. M. Wilbur, *J. biol. Chem.* **174**, 257 (1948).
11. A. Müller and H. Sies, *Meth. Enzymol.* **105**, 311 (1984).
12. A. Boveris, E. Cadenas and B. Chance, *Fedn. Proc. Fedn. Am. Socs. exp. Biol.* **40**, 195 (1981).
13. E. Cadenas and H. Sies, *Meth. Enzymol.* **105**, 221 (1984).
14. G. M. Bartoli, A. Müller, E. Cadenas and H. Sies, *FEBS Lett.* **164**, 371 (1983).
15. B. O. Christophersen, *Biochem. J.* **106**, 515 (1968).
16. R. F. Burk, *Biochim. biophys. Acta* **757**, 21 (1983).
17. K. A. Caldwell and A. L. Tappel, *Biochem.* **3**, 1643–1647 (1964).
18. K. A. Caldwell and A. L. Tappel, *Archs. Biochem. Biophys.* **112**, 196 (1965).
19. K. Yasuda, H. Watanabe, S. Yamazaki and S. Toda, *Biochim. biophys. Res. Commun.* **96**, 243 (1980).
20. A. Wendel, M. Fausel, H. Safayhi, G. Tiegs and R. Otter, *Biochem, Pharmac.* **33**, 3241 (1984).
21. M. J. Parnham and S. Kindt, *Biochem. Pharmac.* **33**, 3247 (1984).

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REVIEW

Molecular Actions of Ebselen—an Antiinflammatory Antioxidant

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(Received 28 November 1994)

Abstract—1. Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) is a non-toxic seleno-organic drug with antiinflammatory, antiatherosclerotic and cytoprotective properties.

2. Ebselen and some of its metabolites are effective reductants of hydroperoxides including those arising in biomembranes and lipoproteins.

3. By reactions with hydroperoxides and thiols several interconversion cycles are formed which include ebselen metabolites with varying oxidation number of the selenium.

4. In the presence of thiols ebselen mimics the catalytic activities of phospholipid hydroperoxide glutathione peroxidase.

5. Ebselen inhibits at low concentrations a number of enzymes involved in inflammation such as lipoxygenases, NO synthases, NADPH, oxidase, protein kinase C and H^+/K^+-ATPase. The inhibitions are manifested on the cellular level and may contribute to the antiinflammatory potential of ebselen.

Key Words: Ebselen, selenium, antioxidant, inflammation, oxidative stress, hydroperoxides, lipid peroxidation, lipoproteins, atherosclerosis, cytoprotection, drug toxicity, free radicals, lipoxygenase, eicosanoids

INTRODUCTION

Many diseases are accompanied or even caused by oxidative stress which is characterised by a situation that more reactive oxygen species are formed than can be counteracted by the antioxidant defence systems of the organism. Therefore the therapeutic use of natural or synthetic antioxidants appears to be promising (Yoshikawa *et al.*, 1993). Among the antioxidants tested during the last decade, superoxide dismutase and xanthine oxidase inhibitors may be of limited relevance for human use, whereas probucol and ebselen are more promising even though their therapeutic efficacy still needs to be confirmed by controlled clinical trials (Halliwell, 1992).

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) is an antiinflammatory seleno-organic compound that has been extensively studied during the last decade. The particular interest in this drug resulted among others from the early observation that ebselen mimics glutathione peroxidase (GPx) activities (Müller *et al.*, 1984, Wendel *et al.*, 1984) in particular that of phospholipid hydroperoxide glutathione peroxidase (Maiorino *et al.*, 1988). In recent reviews on ebselen Sies (1993, 1994) placed the main emphasis on this enzyme-like catalytic property. Indeed, a catalytic activity of a drug being a low-molecular organic compound is particularly surprising, and there is some indication that this pseudoenzymatic activity may contribute in part to the pharmacodynamic profile of ebselen. Considering the mechanism of action of the GPx-like activity of ebselen (see below), it becomes plausible, however, that ebselen displays a unique pattern of chemical reactions which is not restricted to the GPx reaction. The special chemistry of ebselen appears to be the reason for the diversity of the ebselen actions in a great number of *in vitro* and *in vivo* systems as reported in the pertinent literature. The objective of the present review is to compile the molecular actions of this seleno-organic drug in a complex manner and to derive possible implications for its pharmacology.

A substantial part of the pharmacological profile of ebselen appears to be due to its action as antioxidant with a unique mode of action. A number of other antioxidants such as α-tocopherol and probucol act

as scavengers of lipid free radicals and thus prevent lipid peroxidation including secondary reactions triggered by it. In contrast, ebselen is a poor free-radical scavenger if at all but it is an effective scavenger of organic hydroperoxides, in particular of hydroperoxy-lipids. Since the latter products are capable of propagating lipid peroxidation, their removal leads eventually to the same result as in the case of radical scavengers. However, the beneficial effects of radical scavengers, in particular of α-tocopherol, are limited owing to their co-oxidative destruction during action. This phenomenon does not occur with ebselen.

CHEMICAL CONVERSIONS OF EBSELEN

Ebselen is chemically highly reactive, in particular toward hydroperoxides and thiols. In such reactions derivatives are formed which differ from each other in the oxidation number of the selenium moiety. Ebselen itself may be regarded as the inner anilide of an arylselenenic acid, in which the selenium possesses the oxidation number +2. By various redox reactions ebselen can be converted to compounds representing the oxidation states of seleninic acid (+4), selenol (−2), diselenide (−1) and selenenylsulphides (±0). At first sight the pronounced redox activity of ebselen seems to contradict the fact that this drug possesses low toxicity—in contrast to most of other selenium compounds—and a comparatively long biological half-life in man and laboratory animals. However this contradiction may be simply solved by consideration of the fact that ebselen possesses the highest thermodynamic stability among all derivatives. This stability is a consequence of the energetically favoured five-membered ring. By combination of various redox reactions several reaction cycles can proceed in which ebselen is regenerated. Figure 1 gives a survey on these reaction possibilities.

Reaction with hydroperoxides

Ebselen and some of its metabolic derivatives are capable of reducing hydroperoxides to the corresponding hydroxy compounds (Fischer and Dereu, 1987; Morgenstern *et al.*, 1992, Maiorino *et al.*, 1992). As shown in Table 1, ebselen selenol possesses the highest reactivity towards hydrogen peroxide, whereas its selenenylsulphide exhibits only a low reaction rate. Among the organic hydroperoxides, hydroperoxy phospholipids, hydroperoxy cholesteryl esters and cholesterol hydroperoxide are effectively reduced by ebselen (Maiorino *et al.*, 1992). The reductive detoxification of hydroperoxides appears to be a substantial component of the pharmacodynamic activity of ebselen.

The oxidation of ebselen to its selenium oxide can also be accomplished by the flavin-containing monooxygenase from pig liver in an NADPH-dependent reaction (Ziegler *et al.*, 1992). During the catalytic cycle of this enzyme a hydroperoxyflavin intermediate is formed which is the putative oxidant of ebselen. Similar reactions also occur with ebselen selenol and its methylated biotransformation product (Ziegler *et al.*, 1992; Akerboom *et al.*, 1995). In this manner, the reactions of hydroperoxides in the ebselen metabolism can be mimicked by oxygen and the NADPH-dependent flavin-containing monooxygenase system. From experiments with perfused rat liver it was deduced that these reactions may also occur in intact tissues (Akerboom *et al.*, 1995).

Reaction with thiols

Ebselen reacts with a number of thiols such as glutathione, *N*-acetyl-*L*-cysteine, dithiothreitol and dihydrolipoate forming selenenylsulphides (Kamigata *et al.*, 1986b; Cotgreave *et al.*, 1987; Haenen *et al.*, 1990), which in turn are converted in the presence of an excess of thiol to ebselen selenol and ebselen diselenide (see Fig. 1). The occurrence of the selenol as intermediate in the reduction by thiols of the selenenylsulphide to the diselenide was clearly demonstrated by Cotgreave *et al.* (1992). These authors trapped the selenol by reaction with 1-chloro-2,4-dinitrobenzene and identified the conjugate by various spectroscopic methods. Thiols are also capable of reducing the selenium oxide of ebselen (Kamigata *et al.*, 1986c; Fischer and Dereu, 1987; Glass *et al.*, 1989). The precise reaction sequence of the latter conversion is not clear. It remains to elucidate whether the reduction proceeds without (cycle A in Fig. 1) or with ring opening (cycle A′) or both. In the latter two cases it would have to be clarified, which compound undergoes ring opening—ebselen selenoxide or its cyclic thiol adduct. Moreover, presuming cycle A′ some authors proposed the intermediary rearrangement of the seleninylsulphide to the selenenic-sulphenic mixed anhydride R′–S–O–Se–R″ (not shown in Fig. 1) before the cleavage to the selenenic acid by the second thiol molecule takes place (Reich and Jasperse, 1987; Glass *et al.*, 1989). The mechanism of the reaction of ebselen selenoxide with thiols apparently depends also upon the type of the thiol. With α-toluenethiol a thiocarbonyl intermediate could be demonstrated, whereas such a possibility was excluded with other thiols such as *N*-acetyl-*D*,*L*-cysteine (Glass *et al.*, 1989).

Ebselen also reacts with thiol groups of proteins. A variety of enzyme inhibitions may be accounted for by such a reaction (see below). A particular role may be ascribed to the reaction with serum albumin.

Ebselen is covalently bound to serum albumin via a selenenylsulphide bond both *in vitro* (Nomura *et al.*, 1989) and *in vivo* (Wagner *et al.*, 1994). The albumin-bound ebselen can be transferred, however, to other proteins containing reactive thiols such as glutathione S transferases and membrane proteins (Nikawa *et al.*, 1994a). This transfer appears to be crucial for the uptake of ebselen by the cells (Wagner *et al.*, 1994).

Interconversions of ebselen: the GPx-like activity

The discovery of the GPx-like activity of ebselen in 1984 has attracted the interest of many researchers. Since the glutathione peroxidases and ebselen share the presence of a catalytically active selenium, it was plausible that ebselen could be a model for the catalytic mechanism of the GPx. The active site of the GPx contains a selenocysteine which is converted during the catalytic cycle to a selenenic acid derivative and glutathione selenenylsulphide (for review see Flohé, 1989). In the case of ebselen the analogous catalytic cycle must imply the selenol form of ebselen. In earlier work on the mechanism of the GPx-like activity of ebselen Fischer and Dereu (1987) were not able to demonstrate a selenol intermediate. On the basis of their experimental data they proposed the functioning of two catalytic cycles (cycles A and B in Fig. 1) dependent on whether the hydroperoxide (cycle A) or the thiol (cycle B) occurs in excess over the other reaction partner. It was the merit of these authors to demonstrate relevant chemical interconversions of ebselen independent of their involvement in the GPx-like activity. The failure to demonstrate ebselen selenol in this study may be due to the fact that the authors conducted the experiments in an aprotic solvent, dimethylformamide; moreover they used benzylmercaptane instead of glutathione as thiol. Later work of other groups has unequivocally established the transient formation of the selenol in aqueous systems containing glutathione (Maiorino *et al.*, 1988; Haenen *et al.*, 1990; Cotgreave *et al.*, 1992; Morgenstern *et al.*, 1992). In this way the cycle C would be operative under the premise that both ebselen selenol and ebselen diselenide are obligate intermediates. Cycle C was supported by the observation that the rate of the formation of ebselen diselenide from ebselen glutathione selenenylsulphide is linearly dependent on the concentration of glutathione but no net consumption of glutathione ensues (Haenen *et al.*, 1990), However, owing to its high reactivity toward hydroperoxides (Table 1), the selenol can also be directly converted to ebselen, thus closing cycle D. It has been estimated that under conditions of a typical peroxidase assay the selenol is the predominant molecular species responsible for the glutathione-(70%) and dithiothreitol-dependent (96%) peroxidase activities of ebselen via cycle D (Morgenstern *et al.*, 1992).

As mentioned above, interconversion reactions mediated by hydroperoxides can also be brought

Table 1. Second order rate constants of ebselen and its derivatives with hydrogen peroxide*

Compound	Rate constant (mM^{-1} min^{-1})
Ebselen	0.29 ± 0.07
Ebselen glutathione selenenylsulphide	≤ 0.01
Ebselen selenol	2.8 ± 0.5
Ebselen diselenide	0.32 ± 0.05

*From Morgenstern *et al.* (1992).

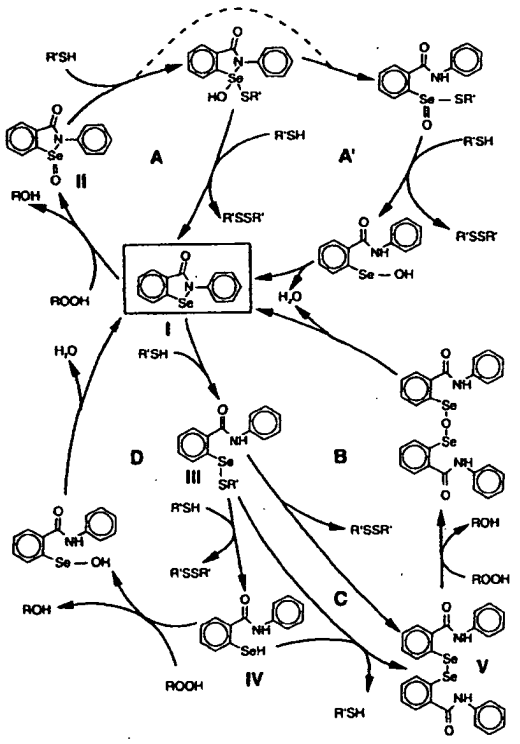

Fig. 1. Interconversions of ebselen and its metabolites by reaction with hydroperoxides and thiols and reaction cycles (A–D) under discussion for the glutathione peroxidase-like activity of ebselen. The compounds are ebselen (I), ebselen selenoxide (II), ebselen selenenylsulphide (III), ebselen selenol (IV) and ebselen diselenide (V). Compounds without a Roman number are putative intermediates. R stands for residues of polyunsaturated phospholipids, cholesteryl esters, cholesterol or any other organic hydroperoxide or hydrogen; R′ stands for glutathione, dihydrolipoate, *N*-acetylcysteine or thiol protein residue. Cycle A is operating if the hydroperoxide is in excess over the thiol; otherwise the cycles B, C or D are preferred. Cycle B may occur in aprotic solvents whereas cycles C and D include compound IV and operate in aqueous systems in proportions dependent on the type of the thiol. Cycle D is analogous to the catalytic cycle of selenium-containing glutathione peroxidases. The scheme does not contain the comproportion reaction (II) + (V) → 3 (I) + H_2O which affords a further possibility to regenreate ebselen from its metabolites. Moreover the exchange reaction of compound III with other thiols is not considered.

about by oxygen in the presence of NADPH and the flavin-containing monooxygenase. The possible role of this system in the ebselen metabolism *in vivo* is supported by the observation that ebselen and its biotransformation product 2-(methylseleno)-benzanilide enhance the efflux of glutathione disulphide in the perfused rat liver which is suppressed by the monooxygenase inhibitor *N*-benzylimidazole (Akerboom *et al.*, 1995). This efflux of glutathione disulphide may be brought about via cycle D in Fig. 1, with the exception that the reoxidation of ebselen selenol is caused by the monooxygenase. It remains in question, however, whether the monooxygenase pathway is implicated in the therapeutic effects of ebselen, inasmuch as an enhanced release of oxidised glutathione from cells may be regarded as a criterion for oxidative stress rather than for an antioxidant or antiinflammatory effect. Conversely, in another experimental model ebselen was shown to protect hepatocytes against drug-induced depletion of glutathione (Li *et al.*, 1994).

Recently another catalytic activity of ebselen related to its redox interconversions has been described, acceleration of the reduction of ferricytochrome c by thiols (Engman *et al.*, 1994). Here again ebselen selenol or its selenolate anion, respectively, may transfer one electron to the cytochrome thereby forming a selenium-centered radical that in turn recombines to ebselen diselenide. In this way, the thiol-cytochrome c reductase mimetic activity may be brought about by redox shuttling between ebselen diselenide and ebselen selenol.

Dihydrolipoate has been shown to be a better cofactor for the GPx-like activity of ebselen as compared to glutathione (Haenen *et al.*, 1990). This difference appears to be due to the presence of a second thiol group which constitutes an intramolecular nucleophile in the corresponding selenenylsulphide so that the latter is more rapidly converted to the selenol and then to the diselenide. From this property of dihydrolipoate some implications as to the pharmacology of ebselen may be considered. Although the intracellular concentration of dihydrolipoate is lower by several orders of magnitude than that of glutathione, this dithiol reacts preferably with ebselen. In this way an accumulation of ebselen glutathione selenenylsulphide *in vivo* is counteracted. The glutathione adduct of ebselen is comparatively more hydrophilic and believed to give rise to a higher renal clearance of ebselen (Cotgreave *et al.*, 1988). For this reason, the pharmacologic efficacy of ebselen is expected to be improved by simultaneous administration of lipoic acid. Lipoic acid alone is a drug used in a variety of diseases including liver and neurological disorders and asthma. *In vivo* it forms a redox couple with dihydrolipoate by the action of mitochondrial keto-acid dehydrogenases. The dihydrolipoate turned out to be a universal free-radical quencher which can scavenge peroxyl radicals both in the cytosol and in the hydrophobic membrane domains (Kagan *et al.*, 1992). In this manner, lipoic acid is an antioxidant prodrug with a mode of action quite different from that of ebselen. Therefore, the two drugs may act synergistically in the therapy of disorders connected with oxidative stress.

The GPx-like activity of ebselen is not only exerted with hydrogen peroxide but also apparently with any organic hydroperoxide. The rate of the reaction with hydroperoxy phospholipids has been reported to be more than 10 times higher than that with hydrogen peroxide (Maiorino *et al.*, 1988). This preference of lipophilic substrates may be due to the hydrophobic nature of the ebselen molecule. Thus, with respect to its catalytic activity ebselen resembles the selenoenzyme phospholipid hydroperoxide glutathione peroxidase (PHGPx) with the distinction that the PHGPx possesses a molecular activity three orders of magnitude higher than that of ebselen (Maiorino *et al.*, 1988). PHGPx has been shown to be widely distributed in rat tissues (Roveri *et al.*, 1994). Owing to the much higher molecular activity of true PHGPx, the PHGPx-mimetic activity of ebselen may be relevant only for such compartments where PHGPx is absent (e.g. in blood plasma) or present in very low concentrations. Moreover, it should be stressed that even in severe selenium deficiency of mice the activity of PHGPx in various tissues only drops to about 30—70% of the control values (Weitzel *et al.*, 1990) which should be still sufficiently high to sustain the enzymatic reduction of hydroperoxy-lipids in cells unless extremely severe oxidative stress proceeds. These considerations give rise to some doubt whether the multiple pharmacological actions of ebselen, in particular those displayed on the cellular level, can be explained solely by the GPx-like activities. Rather it appears likely that partial routes of the reaction cycles shown in Fig. 1 contribute to many actions of ebselen. A GPx-like activity operates only in such cases in which one of the cycles A, B, C or D is repeatedly closed. Thus, the pharmacology of ebselen may be primarily the consequence of its complex redox interconversion chemistry, a reflection of which are among others the GPx-like activities.

Photochemistry of ebselen

The photochemical reactions of ebselen need to be only considered if this drug is intended to be used in cutaneously administered pharmaceutical compositions or cosmetics (e.g. for the treatment of U.V.

erythema or in sun protectants). The photochemistry of ebselen was studied by Kamigata *et al.* (1986a). These authors observed multifarious conversions upon irradiation of ebselen in the UVB range. The Se–N bond in ebselen turned out to be particularly sensitive to homolytic cleavage. The intermediate *bis*-radical recombines readily to a seven-membered seleno-lactame ring; the corresponding compound could be isolated from the reaction sample in benzene in high yield. When the authors the irradiation performed in *t*-butanol, they found a variety of additional reaction products including ebselen selenoxide which was believed to be a photooxidation product under these conditions. The proposed reaction sequences are shown in Fig. 2. Under aerobic conditions the putative peroxo and hydroperoxo radicals of ebselen may be expected. These radicals are believed to be highly reactive. In particular, the ebselen hydroperoxo radical shares some reaction possibilities with hydroxyl radicals, so that it may be regarded as crypto-OH radical. It is conceivable that identical radicals like those in photooxidation are also formed by reaction of peroxyl radicals with ebselen. This possibility would explain the marked prooxidative behaviour of ebselen in certain free-radical model systems (see next chapter).

Other reactions of ebselen

Ebselen selenol appears to play a role as intermediate not only in the interconversions of ebselen but also in its hepatic biotransformations. This conclusion arises from the structures of the urinary and plasma metabolites which represent Se-methylated and Se-glucuronylated derivatives of ebselen selenol. The Se-methylated ebselen selenol (2-[methylseleno]-benzanilide) is believed to display still some pharmacologic activity since it can be converted to the corresponding selenoxide (Akerboom *et al.*, 1995). For details as for the biotransformations of ebselen the reader is referred to the former reviews by Sies (1989) and Sies (1993).

Ebselen has been reported to convert a defined proportion of rat liver cytochrome *P*-450 in a time-dependent reaction (Kühn-Velten and Sies, 1989). A half-maximal effect was observed with 50 μM ebselen. Pretreatment of cytochrome *P*-450 with dithioerythritol or bovine serum albumin prevented this conversion. The authors proposed that only certain isoenzymes may be susceptible to this effect of ebselen and that ebselen reacts with the thiolate anion of the apoprotein being the fifth ligand of haem iron.

Furthermore it was demonstrated that ebselen converts leukotriene B_4 to its biologically inactive 6-*trans* isomer (Kuhl *et al.*, 1986). The mechanism of this isomerization, that could be a further catalytic action, has not been studied.

EBSELEN AND LIPID PEROXIDATION

Reactions with free radicals

It has been shown by pulse radiolysis experiments that ebselen reacts rapidly with trichloromethylperoxyl radical, the rate constant being $2.9 \times 10^8\ M^{-1}\ sec^{-1}$, and other halogenated radicals (Schöneich *et al.*, 1990). Indirect evidence was also obtained for a reaction with non-halogenated peroxyl radicals by the use of a carotinoid bleaching competition test (Maiorino *et al.*, 1992). In contrast, ebselen does not react with diphenylpicrylhydrazyl that is reactive against potent free-radical scavengers (Noguchi *et al.*, 1992). It follows from these data that ebselen is capable of reacting only with highly reactive radicals, but even in the case of peroxyl radicals this reaction is not a radical-scavenging one, since the carotinoid bleaching reaction was shown to be enhanced by ebselen rather than inhibited (Maiorino *et al.*, 1992). It may be speculated that ebselen forms with peroxyl radicals a selenium peroxo-*bis* radical which is more reactive than the parent radical (see left part of Fig. 2).

The lack of radical-scavenging activity of ebselen is further substantiated by the observation that ebselen does not inhibit lipid peroxidation induced by free-radical initiators such as 2,2′-azo-*bis*-(amidino-propane) and that it does not protect α-tocopherol from co-oxidative destruction during this process (Noguchi *et al.*, 1992).

A matter of speculation is also the putative reaction of ebselen with superoxide or hydroperoxyl radicals. Cotgreave *et al.* (1989) observed that ebselen, when applied in concentrations lower than those needed for inhibition of the NADPH oxidase system of granulocytes, caused in phorbol ester-stimulated granulocytes a reduction of external acetylated cytochrome c which was not inhibited by superoxide dismutase excluding the involvement of free superoxide. This reduction may have been caused by a superoxide adduct of ebselen which is supposed to be prone to oxidation forming ebselen and oxygen.

Ebselen is also capable of quenching singlet oxygen with a rate constant of $2.5 \times 10^6\ M^{-1}\ sec^{-1}$ (Scurlock *et al.*, 1991). As compared to other singlet oxygen quenchers this activity is however only modest. All in all, the reactions of ebselen with free radicals appear to be—if any—of minor importance for the pharmacological actions of this drug.

Action on lipid peroxidation

As mentioned above ebselen fails to inhibit lipid peroxidation induced by free-radical initiators. By

contrast, ebselen in micromolar concentrations is a potent inhibitor of lipid peroxidation processes induced by transition metals, e.g. in microsomes (Müller *et al.*, 1984), in mitochondria (Narayanaswami and Sies, 1990) and with methyl linoleate (Noguchi *et al.*, 1992). This type of lipid peroxidation is brought about by a Fenton-type reaction of the metal ion with traces of hydroperoxides forming an alkoxyl radical and the higher valency state of the metal; the alkoxyl radical in turn propagates a free-radical chain reaction. Ebselen inhibits this process at its earliest stage by removing the hydroperoxides. It should be

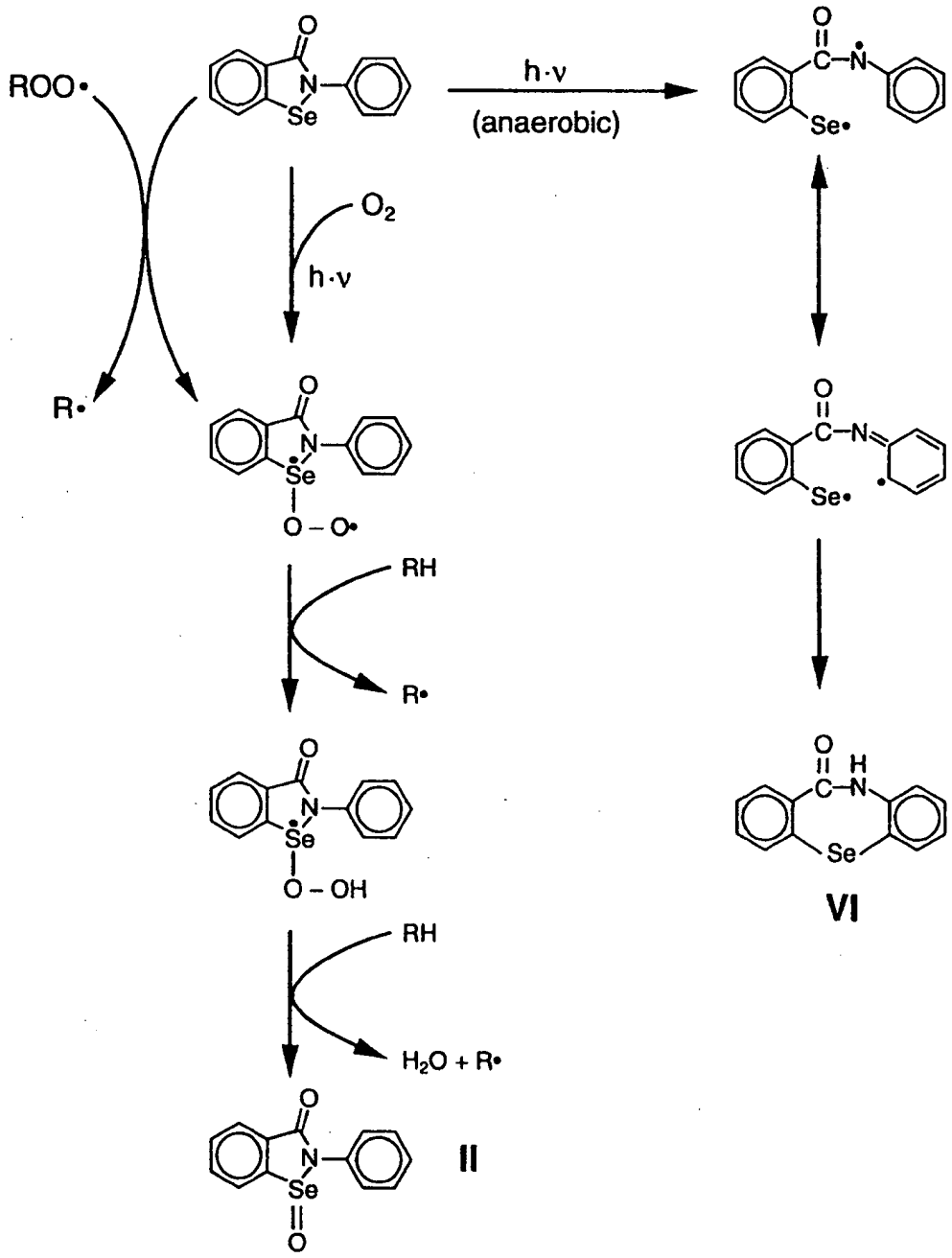

Fig. 2. Photochemical conversions of ebselen under aerobic and anaerobic conditions. In the absence of oxygen, there occurs a homolytic cleavage of the Se–N bond leading to a *bis*-radical that recombines to the seleno-lactame VI. In the presence of oxygen, photooxidation of ebselen yields the putative peroxo and hydroperoxo radicals that oxidise other compounds thereby being reduced to ebselen selenoxide (II) as one of the final photooxidation products. The secondary radicals R· react with either ebselen or compound VI or with the photolysis products of them, so that a number of other stable products can be formed (not shown here). For details see Kamigata *et al.* (1986a). The peroxo radicals of ebselen may also be formed by reaction of peroxyl radicals with ebselen.

stressed that under certain conditions ebselen and its analogues may even stimulate lipid peroxidation as was observed with hepatocytes in which lipid peroxidation was initiated by diquat redox cycling (Andersson *et al.*, 1994). Hence ebselen cannot be regarded as a universal inhibitor of lipid peroxidation.

The inhibition by ebselen of certain forms of lipid peroxidation is not obligatorily dependent on the presence of glutathione (Müller *et al.*, 1984) indicating that the hydroperoxide-reducing action rather than the GPx-like activity is responsible for the inhibition. Glutathione is however required in such *in vitro* systems in which the formation of hydroperoxy-lipid exceeds the concentration of ebselen available. In this case glutathione is needed to regenerate the ebselen from ebselen selenoxide (cycle A in Fig. 1). Such a situation should be however rarely met *in vivo*, inasmuch as even in oxidative stress the accumulation of hydroperoxides is to a large extent counteracted by the physiological defence systems such as glutathione peroxidases. Moreover, it must be stressed that the study of the interaction of ebselen and glutathione in lipid peroxidation processes is hampered by the fact that glutathione is an antioxidant by itself, possibly by virtue of scavenging free radicals. In this way synergistic effects of glutathione and ebselen independent of the GPx-like activity may explain the observation of Müller *et al.* (1985) that ebselen fails to inhibit the ferrous ADP-induced lipid peroxidation in glutathione-depleted hepatocytes obtained from phorone-treated rats.

Effect on oxidative modification of lipoproteins

Oxidative modification of low-density lipoproteins (LDL) is thought to be an early event in the pathogenesis of atherosclerosis in man (Steinberg *et al.*, 1989). This process can be brought about by either hydroperoxy-lipids or reactive oxygen species that oxidise lysine residues in apoprotein B-100, which gives rise to a preferential uptake of the lipoprotein by monocyte-derived macrophages via scavenger receptors. Studies with cholesterol-fed rabbits supported the idea of the possible involvement of 15-lipoxygenase in the oxygenation of LDL and in the development of atherosclerotic lesions (Kühn *et al.*, 1994). The oxygenation of the lipid compartment of the LDL concerns to a large extent the cholesteryl esters. However in blood plasma there is no effective physiological system for the removal of the hydroperoxides of cholesterol and cholesteryl esters residing in lipoproteins. Ebselen has been reported to effectively reduce these hydroperoxides to the corresponding alcohols (Maiorino *et al.*, 1992; Sattler *et al.*, 1994) and to inhibit the copper-induced oxidative modification of LDL (Thomas and Jackson, 1991; Noguchi *et al.*, 1994). The latter conclusion is based on the observations that ebselen inhibits the oxygen uptake and maintains the intactness of the apoprotein. The copper-induced oxidative modification is believed to require the presence of trace amounts of hydroperoxy-lipids in the lipoproteins (Thomas and Jackson 1991); its removal by ebselen provides efficient protection against this deleterious action. Moreover, ebselen reduces dramatically the cytotoxic action of oxidatively modified LDL against endothelial cells (Thomas *et al.*, 1993). Therefore ebselen could be a potential antiatherosclerotic drug. This proposal has to be confirmed, however, in experimental studies with laboratory animals and in clinical trials.

A conflicting issue appears to be the state and activity of ebselen in plasma and whole blood respectively. As stated before, ebselen seems to be to a large part covalently bound to serum albumin via a selenenylsulphide bond. This albumin-bound ebselen is not capable of reducing the hydroperoxides present in oxidatively modified LDL in the absence of cells (Christison *et al.*, 1994). Since this interaction between the two high-molecular complexes could be sterically hindered, testing of the reducing capacity of albumin-bound ebselen with low-molecular hydroperoxides such as H_2O_2 is warranted but not reported so far. A further complication is the fact that the concentrations of glutathione and other thiols in the plasma are normally very low so that it is uncertain whether the GPx-like activity of ebselen can operate in the plasma. It was reported, however, that preincubation of human plasma with ebselen concentrations as low as 1 μM strongly reduced the level of hydroperoxy-phosphatidylcholine (Miyazawa *et al.*, 1993). The same authors also found that oral administration of ebselen to rats gave rise to a dose-dependent marked lowering of the plasma level of this hydroperoxy-lipid within 2–4 h whereas the liver and red cell levels were not affected. These observations suggest a preferential hydroperoxide-reducing activity in plasma. Stocker and coworkers (Christison *et al.*, 1994) observed that the hydroperoxy cholesteryl esters in oxidatively modified plasma lipoproteins are reduced by ebselen in whole blood but not in plasma suggesting that the reduction is cell-mediated. It remains to be clarified whether the blood cells release cofactors required for the ebselen action, e.g. low-molecular thiols, or whether there occurs an interaction of the plasma form of ebselen with membrane constituents, e.g. ecto-thiol groups. Irrespective of this problem the experimental data available argue in favour of the assumption that plasma ebselen is an effective reductant of peroxidized plasma lipoproteins

in vivo and that ebselen may display its antioxidative capacity preferably in blood plasma, where sizeable glutathione peroxidase activity with peroxidized lipoproteins as substrate is apparently missing (Christison *et al.*, 1994) in contrast to the cells that contain PHGPx. The nature of the reducing ebselen species in the plasma is however far from clear. The selenenylsulphides of albumin or other thiol proteins are possible candidates, but this assumption is contradicted by the fact that ebselen-glutathione selenenylsulphide exhibits only low reactivity toward hydrogen peroxide (Morgenstern *et al.*, 1992). It cannot be excluded, however, that the selenenylsulphides of thiol proteins and glutathione differ from each other in their redox potentials leading to different reactivities. Although the additional existence of non-covalent forms of ebselen binding to serum albumin, e.g. by the hydrophobic binding domain as with free fatty acids, cannot be ruled out, the pharmacology and biotransformation of ebselen can be satisfactorily explained with the selenenylsulphide adduct of ebselen with serum albumin being the transport form in plasma. This assumption is corroborated by the recent work of Wagner *et al.* (1994) who demonstrated an excellent bioavailability of this adduct.

EBSELEN AS A MULTIPLE ENZYME INHIBITOR

General considerations

Ebselen has been reported to inhibit at low concentrations a variety of enzymes (Table 2). The majority of these enzymes are implicated in inflammatory processes so that it is reasonable to assume that the inhibitory effects may contribute to some extent to the antiinflammatory actions of ebselen *in vivo*. This assumption is however contradicted by the fact that all of these inhibitory effects—so far examined—are prevented or reversed by glutathione or other thiols. In the case of mammalian 5- and 15-lipoxygenases, gastric H^+/K^+-ATPase, nitric oxide synthase, protein kinase C and leukocyte NADPH oxidase the inhibitory effects were also demonstrated on the cellular level, but with intact cells much higher concentrations of ebselen are required to achieve comparable inhibitions as with the pure enzymes. The lower sensitivity of these enzymes in cells appears to be due to the counteractive effect of glutathione as well as to the binding of ebselen to other proteins.

Lipoxygenases

The inhibition of 5-lipoxygenase is of particular interest because evidence has been obtained that ebselen suppresses *in vivo* the formation of pro-inflammatory cysteinyl leukotrienes (Wendel and Tiegs, 1985; Tabuchi *et al.*, 1995) synthesized via the 5-lipoxygenase pathway of arachidonic acid metabolism. While the pure enzyme is strongly inhibited by an ebselen concentration as low as 0.1 μM (Schewe *et al.*, 1994), the formation of 5-lipoxygenase products in polymorphonuclear leukocytes is inhibited at concentrations of ebselen as high as 20 μM (Safayhi *et al.*, 1985). Such concentrations were observed however in blood plasma after oral administration of ebselen. Two modes of action may be considered to explain the lipoxygenase inhibition in cells: directly

Table 2. Inhibitory effects of ebselen on enzymes

Enzyme	Species, tissue	Preparation	IC_{50} (μM)	Reference
15-Lipoxygenase	Rabbit reticulocytes	Pure enzyme	0.17*	Schewe *et al.* (1994)
5-Lipoxygenase	Human recombinant	Pure enzyme	< 0.1	Schewe *et al.* (1994)
Prostaglandin H synthase I	Sheep vesicular glands	Microsomal membranes	38	Schewe *et al.* (1994)
Nitric oxide synthase (constitutive)	Bovine aorta endothelium	Cell homogenate	8.5* 13	Zembowicz *et al.* (1993) Hattori *et al.* (1994)
Nitric oxide synthase (inducible)	Rat peritoneal macrophages	Cytosol	2.5	Hattori *et al.* (1994)
NADPH oxidase	Human granulocytes	Membrane pellet	0.5–1.0*	Cotgreave *et al.* (1989)
NADPH-cytochrome *P*-450 reductase	Mouse liver	Microsomal membranes	0.13	Wendel *et al.* (1986)
NADPH-cytochrome *P*-450 reductase	Rat liver	Microsomal membranes	0.55	Nagi *et al.* (1989)
NADH-cytochrome b_5 reductase	Rat liver	Microsomal membranes	0.2–0.3	Nagi *et al.* (1989)
Protein kinase C	Human granulocytes	Partially purified enzyme	0.5*	Cotgreave *et al.* (1989)
H^+/K^+-ATPase	Pig stomach	Leaky gastric membranes	0.15* 0.06*	Beil *et al.* (1990) Tabuchi *et al.* (1994)
Glutathione S-transferase	Rat liver	Isolated isoenzymes	~50*†	Nikawa *et al.* (1994b)
Papain	Papaya latex	Pure enzyme	*	Nikawa *et al.* (1994b)

*Inhibition prevented or reversed by glutathione.
†Time-dependent inhibition.

by formation of an enzyme–ebselen complex and indirectly by lowering the hydroperoxide tone. The latter phenomenon is based on the fact that lipoxygenases require a certain level of hydroperoxy fatty acids in the micromolar range to start their catalytic cycle. Therefore compounds that reduce hydroperoxides are generally capable of inhibiting lipoxygenase reactions. The possible relevance of the two modes of lipoxygenase-inhibitory actions of ebselen has been studied with pure 15-lipoxygenase from rabbit reticulocytes (Schewe *et al.*, 1994). From the analysis of the reaction products of this enzyme under various conditions of partial inhibition by ebselen the following conclusions have been drawn: (1) in the absence of glutathione ebselen inhibited directly with an IC_{50} of 0.17 μM; (2) in the presence of glutathione ebselen inhibited by lowering the hydroperoxide tone with an IC_{50} of 234 μM; with ebselen glutathione selenenylsulphide a comparable inhibitory potency was observed suggesting the involvement of the GPx-like activity under these conditions. However, in interleukin-4-stimulated monocytes the 15-lipoxygenase was inhibited by ebselen with an IC_{50} of about 20 μM (Heydeck and Schewe, 1994 unpublished) which is difficult to reconcile with the aforementioned data on the pure enzyme in the presence of glutathione. This discrepancy may be due to the fact that in cells the lipoxygenases are regulated via the hydroperoxide tone; in this regulation the PHGPx is instrumental (Weitzel and Wendel, 1992). It is conceivable that the inhibition of the lipoxygenases in cells is brought about by a combined hydroperoxide-reducing action of PHGPx and ebselen. Another possibility would be that the inhibition of lipoxygenases by ebselen in intact cells is due to the formation of ebselen diselenide which seems to be metabolically more stable than the glutathione selenenylsulphide. With a structurally related diselenide an IC_{50} value around 5 μM was observed for the inhibition of pure 15-lipoxygenase (Schewe *et al.*, 1994).

Furthermore it has been reported that ebselen plus glutathione do not protect the 5-lipoxygenase against spontaneous aerobic inactivation during enzyme isolation, whereas glutathione peroxidase affords such a production (Zhang *et al.*, 1994).

The fact that the sensitivity of the 15-lipoxygenase toward ebselen is much lower in cells, i.e. in the presence of 2–8 mM glutathione, than extracellularly where the glutathione concentration is only in the micromolar range, may imply a special pharmacodynamic quality of this ebselen effect. As mentioned in the chapter before, the 15-lipoxygenase is possibly involved in the oxidative modification of low-density lipoproteins (LDL) producing the atherogenic form of them. This assumption is corroborated by a co-localization of 15-lipoxygenase and oxidatively modified LDL in atherosclerotic lesions (Ylä-Herttuala *et al.*, 1990). In this lesions production of reactive oxygen species and damage to cells also occur. It is therefore conceivable that in atherosclerotic lesions the 15-lipoxygenase is released from damaged cells (monocyte-derived macrophages, endothelial cells) whereas glutathione is at the same time oxidized without being regenerated. This scenario would selectively enhance the sensitivity of the 15-lipoxygenase toward ebselen in the lesions. In this way ebselen may selectively suppress the deleterious actions of this enzyme without affecting its physiological functions in intact cells—a property that is desired for any drug. Together with the capacity of ebselen to reduce the hydroperoxy-lipids in oxidatively modified LDL (see above) the 15-lipoxygenase-inhibitory effect affords a biochemical basis for the preventive and therapeutic application of ebselen in atherosclerosis which should warrant corresponding experiments with laboratory animals or clinical trials that have not been reported so far. The proposed antiatherosclerotic action of ebselen is illustrated in Fig. 3.

The lipoxygenase-inhibitory activities of ebselen could also play an important role in the protection against the unwanted restenosis occurring upon percutaneous transluminal coronary angioplasty (PTCA). The PTCA is a modern and widely used

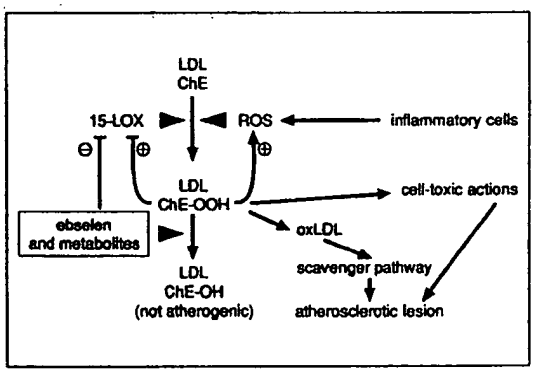

Fig. 3. The role of oxidative modification of low-density lipoproteins (LDL) in the formation of atherosclerotic lesions (simplified) and the counteractions of ebselen. The cholesteryl esters (ChE) in LDL are dioxygenated to hydroperoxides (ChE-OOH) by either 15-lipoxygenase (15-LOX; from endothelial cells or interleukin 4-stimulated monocytes) or reactive oxygen species (ROS). The ChE-OOH give rise to oxidative modification of the apoprotein forming oxLDL, which are no longer taken up via the well-regulated LDL receptor pathway but via the alternate scavenger receptor pathway leading to atherosclerotic lesions. Ebselen suppresses this process in a dual manner by inhibiting 15-LOX and reducing ChE-OOH to the corresponding alcohols (ChE-OH). The full triangles mark chemical conversions; plus and minus designate stimulatory and inhibitory effects, respectively.

method of reperfusing coronary arteries in myocardial infarctions. Since the PTCA necessarily gives also rise to an injury of the arterial endothelium, processes similar to those occurring in the atherogenesis may be induced. In other terms, restenosis and formation of atherosclerotic lesions appear to be closely related. Moreover it has been reported (Serhan and Brezinski, 1991) that PTCA triggers the intraluminal release of cysteinyl leukotrienes (products of 5-lipoxygenase) and of lipoxin A_4 (a product of both 5- and 15-lipoxygenase) *in vivo*, so that it is tempting to speculate that these pro-inflammatory lipoxygenase metabolites contribute to the accelerated restenosis. The therapeutic use of ebselen as an inhibitor of restenosis was claimed in a patent of the Daiichi Pharmaceutical Co. Ltd (Kodama *et al.*, 1993).

NADPH oxidase and protein kinase C

The NADPH oxidase system of phagocytosing cells is instrumental in their microbicidal activity. In a concerted action with superoxide dismutase, myeloperoxidase and lactoferrin a variety of reactive metabolites such as hydroxyl radicals, hypochlorous acid and chloroamines are formed that are capable of killing bacteria and other ingested cells. These reactive metabolites also contribute, however, to inflammatory processes. The NADPH oxidase system is dormant in resting cells and is activated by factors inducing phagocytosis. The activation is a complex signal transduction cascade in which the protein kinase C is included. Protein kinase C is activated by diacylglycerols or phorbol esters, and by calcium and is thought to catalyse the last step of the activation cascade, the phosphorylation of the NADPH oxidase. Ebselen has been shown to suppress the NADPH oxidase system in granulocytes in a dual manner (Cotgreave *et al.*, 1989). Firstly, it counteracts the activation by inhibiting protein kinase C. Other protein kinases of the granulocyte appear to be insensitive toward ebselen. Secondly, ebselen inhibits the enzymic activity of the NADPH oxidase. The two actions of ebselen were established both on the cellular level and with isolated enzyme preparations.

Since protein kinase C exerts multiple functions in inflammation, its inhibition may contribute to the general antiinflammatory potential of ebselen. Thus, the inactivation of lipomodulins leading to activation of phospholipase A_2 and to an enhanced eicosanoid production should be prevented.

H^+/K^+-ATPase

The possible implication of the potent inhibition of the gastric H^+/K^+-ATPase in the gastroprotective actions of ebselen is corroborated by the observation that intraduodenal ebselen suppressed in a dose-dependent manner the gastric secretion in pylorus-ligated rats (Tabuchi and Kurebayashi, 1993). Since in laboratory animals ebselen also counteracts the gastric injury induced by either hydrochloric acid or ethanol, apparently by virtue of its hydroperoxy-lipid lowering and cysteinyl leukotriene synthesis-inhibitory activities (Kurebayashi *et al.*, 1989; Tabuchi *et al.*, 1995), the well-established anti-ulcer effect of ebselen appears to be the result of several combined molecular actions. The failure to inhibit the synthesis of the gastroprotective eicosanoid prostaglandin E_2 (see below) may be advantageous in this respect as well.

Studying the mechanism of the inhibition of the porcine gastric H^+/K^+-ATPase, Tabuchi *et al.* (1994) found that ebselen inhibits both of the two consecutive reaction steps (phosphorylation of the enzyme, K^+-dependent phosphatase) with the latter being more sensitive, as well as the conformational change of the enzyme during catalysis. With respect to this mode of action, ebselen resembles to the gastric drugs omeprazole and SCH28080 but surpasses them as to the inhibitory potency.

Nitric oxide synthases

The inhibition of nitric oxide formation by ebselen was first observed on the cellular level in experiments with rat Kupffer cells (Wang *et al.*, 1992). In experiments with isolated nitric oxide synthases it was evident that the inducible form of the enzyme is more sensitive than the endothelial constitutive one (Hattori *et al.*, 1994). The inducible form is involved in inflammatory processes, e.g. by producing the dangerous peroxynitrite, whereas the constitutive enzyme plays an important physiological role in regulating the vascular tone and circulation (Moncada and Higgs, 1993). For this reason, this selectivity appears to be advantageous for the anti-inflammatory pharmacologic profile of ebselen. Carboxyebselen, a more hydrophilic congener of ebselen, displays a changed selectivity toward various nitric oxide synthases (Hatchett *et al.*, 1994).

Prostaglandin H synthase

Contrary to earlier suggestions in the literature, ebselen is not an inhibitor of the prostaglandin synthesis. The membranous prostaglandin H synthase-1 from sheep vesicular glands is only inhibited by comparatively high concentrations, but this inhibitory effect is overlapped by a pronounced protection of the enzyme from suicide inactivation (Schewe *et al.*, 1994). The inhibition of prostaglandin H synthase in human platelets reported earlier

(Kuhl *et al.*, 1985) may be a secondary effect resulting from the suppression of agonist-triggered rise of intracellular calcium in platelets (Brüne *et al.*, 1991; Dimmeler *et al.*, 1991). The failure to inhibit prostaglandin synthesis has been suggested to be a reason for the lack of protection by ebselen in kidney transplantation in rabbits despite a pronounced antioxidative effect (Gower *et al.*, 1992). On the other hand, the maintenance of the production of prostaglandin E_2 which possesses gastroprotective, bronchodilating and immunosuppressive properties may also be beneficial for the therapeutic use of ebselen. This conclusion is corroborated by the findings that unlike prostaglandin H synthase inhibitors, ebselen does not evoke gastric irritation in rats and, on the contrary, affords protection against the gastric intolerance induced by such drugs (Leyck and Parnham, 1990).

Mechanism of enzyme inhibitions

In many cases the molecular mechanism of the inhibitory actions of ebselen may be a blockade of thiol groups essential for structure and activity of these enzymes. Such a mechanism is however not plausible in the case of mammalian lipoxygenases since they are thought not to contain an essential thiol. From EXAFS spectroscopic studies on rabbit 15-lipoxygenase it was evident that ebselen affects the non-haem iron ligand sphere (Wiesner and Nolting, 1994, unpublished). It remains to be clarified whether ebselen interacts directly with the enzymic iron or whether a thiol group in the vicinity of the active site is covalently modified in such a way that the space-filling ebselen selenenylsulphide residue disturbs one of the iron ligands in the lipoxygenases. It should be emphasized that a protection or reversal by glutathione—as always observed so far examined—does not in any case imply the involvement of a reaction of ebselen with enzymic thiols. Rather such an observation primarily indicates the lack of inhibitory potency for ebselen glutathione selenenylsulphide.

IMPLICATIONS FOR THE PHARMACOLOGY OF EBSELEN

Unlike other selenium compounds, ebselen possesses an extraordinarily low toxicity which is due to the fact that the selenium moiety is not liberated during biotransformation and therefore does not enter the selenium metabolism of the organism (Parnham and Graf, 1987).

The fact that more than 90% of plasma ebselen were shown to be covalently bound to serum albumin *in vivo* (Wagner *et al.*, 1994) is obviously not a barrier as to the bioavailability of the drug for the cells. Surprisingly Wagner *et al.* (1994) did not observe any difference in the uptake of free and albumin-bound labelled ebselen by hepatocytes. The radioactivity could be detected in all subcellular fractions. It must be concluded, therefore, that the transfer of ebselen from the albumin selenenylsulphide to other thiol groups present in plasma membrane proteins is very effective and enables ebselen to permeate easily from the plasma to all compartments of the cell, possibly via a chain of thiol exchange reactions. Since the ebselen selenenylsulphides can enter the interconversion cycles shown in Fig. 1, they can be converted to free ebselen, if hydroperoxides are present. In this manner, the thiol protein-bound ebselen is apt to be a storage form of the drug which is specifically activated under conditions of oxidative stress that is accompanied by an elevated formation of hydroperoxides.

The beneficial pharmacological activities of ebselen have been established in a large number of *in vitro* models and of studies with laboratory animals (Parnham *et al.*, 1991; Parnham and Graf, 1991; Sies, 1993). Most of them appear to be related to the aforementioned molecular actions as well as additional actions on the cellular level that all together constitute a pharmacological profile that is unique for this drug.

Cellular actions of ebselen

In Tables 3 and 4 a selection of cellular *in vitro* actions of ebselen observed by various investigators is compiled. A number of them are related to inflammatory processes and may be accounted for by the enzyme inhibitions described in the foregoing chapter. This holds for the inhibitions of leukotriene B_4 formation, of superoxide production and oxidative burst, as well as of nitric acid formation. Other actions of ebselen are directed to signal transduction processes and to cell–cell interactions. The suppression of the intracellular calcium release and of the corresponding consecutive processes as observed by Brüne *et al.* (1991), has been shown to be attributed to a blockage of the receptor for 1,4,5-inositol-*tris*-phosphate (IP_3) (Dimmeler *et al.*, 1991). The molecular mechanism of this action seems to involve the reaction with an essential thiol. The modulation of the calcium homeostasis by ebselen may also be one reason for the inhibition of eicosanoid syntheses which depend on the activition by calcium of phospholipases. Since the calcium homeostasis differs among various types of cells, it becomes plausible why ebselen affects the eicosanoid formation (prostaglandins as well as 12-hydroxyeicosatetraenoic acid) in various cells and tissues in quite a different manner as observed in the study of Hurst *et al.* (1989).

Table 3. Antiinflammatory actions of ebselen in cellular systems

Cell	Species	Parameter being inhibited	IC_{50} (μM)	Reference
Granulocytes	Rat	Leukotriene B_4 formation	20	Safayhi *et al.* (1985)
Granulocytes	Man	Oxidative burst; $\cdot O_2^-$-production	25	Cotgreave *et al.* (1989)
Kupffer cells	Rat	$\cdot O_2^-$-production	10	Wang *et al.* (1992)
Granulocytes	Man	Adhesion to umbilical vein endothelium	40	Issekutz and Lopes (1992)
Granulocytes	Man	Transendothelial migration	28	Issekutz and Lopes (1992)
Lymphocytes	Man	Mitogenesis	17	Hunt *et al.* (1991)
Lymphocytes	Man	IFN-γ and TNF-α production*		Inglot *et al.* (1991, 1992) Cembrzynska-Nowak and Inglot (1992) Piasecki *et al.* (1992)
Kupffer cells	Rat	NO formation	3	Wang *et al.* (1991)
Platelets	Man	Ca^{++}-release	14	Brüne *et al.* (1991)
Platelets	Man	Arachidonic acid-stimulated cell aggregation	2	Brüne *et al.* (1991)
Platelets	Man	Thrombin-stimulated cell aggregation	9	Brüne *et al.* (1991)
Platelets	Rabbit	Eicosanoid formation	5–9	Hurst *et al.* (1989)

*Induction.

Ebselen has been also found to inhibit the adhesion of polymorphonuclear leukocytes to the endothelium as well as transendothelial migration both *in vitro* (Issekutz and Lopes, 1992) and *in vivo* (Gao and Issekutz, 1993a, b; Gao and Issekutz, 1994). Strong inhibition of chemotaxis and chemokinesis by ebselen *in vitro* was only observed when the migration of leukocytes was induced by leukotriene B_4 but not by chemotactic peptides (Patrick *et al.*, 1993). Therefore it is reasonable to assume that this inhibition is due to the capacity of ebselen to isomerize leukotriene B_4 to its inactive *trans*-isomer (Kuhl *et al.*, 1986).

A further antiinflammatory action of ebselen, the inhibition of mitogenesis in lymphocytes (Hunt *et al.*, 1991), appears to be related to its antioxidant capacity. In great contrast to the latter observation are the findings of Inglot and coworkers that ebselen and ebselen diselenide are mitogens by virtue of the capability of inducing interferon-γ and tumour necrosis factor α (Inglot *et al.*, 1990, 1992; Czyrski and Inglot, 1991; Cembrzynska-Nowak and Inglot, 1992; Piasecki *et al.*, 1992). This effect was reported to be species-specific for man; its pharmacological importance deserves further study.

Ebselen was shown to suppress the contractile responses of guinea-pig lung strips induced by histamine or potassium chloride (Leurs *et al.*, 1990) as well as those of hepatic vasculature upon ethanol-induced damage (Oshita *et al.*, 1994). These observations suggest broncho- and vasodilatory actions that may be brought about by modulating signal transduction processes, perhaps by inhibition of protein kinase C.

Beyond the actions of ebselen related to inflammation (Table 3), a number of cytoprotective actions have been reported (Table 4). The cytoprotection is afforded not only against hydroperoxides but also against the adverse side effects of a variety of drugs,

Table 4. Cytoprotective actions of ebselen in cellular systems

Cell	Species	Noxious agent	Parameter	IC_{50} (μM)	Reference
Endothelial cells	Beef	Hydroperoxy fatty acid	^{51}Cr-release	5	Ochi *et al.* (1992)
Leukaemia L1012 cells	Mouse	*t*-Butyl hydroperoxide	Cell viability	5–10	Geiger *et al.* (1993)
Hepatocytes	Rat	Fe^{++}/ADP	Lipid peroxidation	5–10	Müller *et al.* (1985)
Hepatocytes	Rat	Diquat	Lipid peroxidation, cell viability	~50	Cotgreave *et al.* (1987)
Hepatocytes	Mouse	Paracetamol	Covalent drug binding; enzyme release	~50	Harman *et al.* (1992)
Hepatocytes	Rat	Paracetamol	Enzyme release; lipid peroxidation; depletion of glutathione	~50	Li *et al.* (1994)
LLC-PK1 cells	Mouse	Cisplatin	Cell viability	5–15	Baldew *et al.* (1992)
Leukaemia L1012 cells	Mouse	Merocyanine 540	Cell survival photoinactivation of Na^+/K^+-ATPase	5–10	Lin *et al.* (1992, 1994)
Tumour cells (EMT6)		Mitomycin C	Cell survival	~50	Gustafson and Pritsos (1991)
Tumour cells (MCF-7)	Man	Doxorubicin	Clonogenicity	~5	Doroshow (1986)
Malaria-infected red cells	Man, Mouse	*Plasmodium falciparum* and *P. berghei*	Parasite proliferation	10–15	Hüther *et al.* (1989)

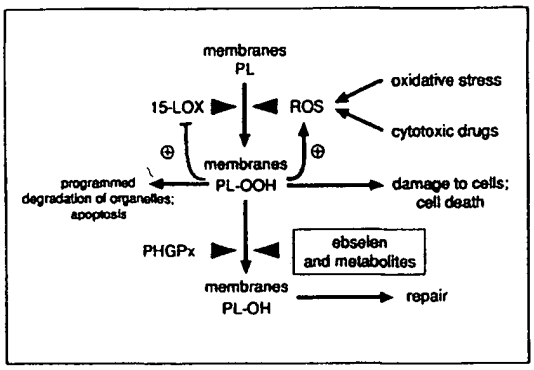

Fig. 4. The cell-protective action of ebselen. Oxidative stress or certain cytotoxic drugs produce reactive oxygen species (ROS) which in turn cause lipid peroxidation in membranes. The hydroperoxy-phospholipids thus formed (PL-OOH) are deleterious for the cell; moreover they trigger the formation of further ROS and activate the 15-lipoxygenase (15-LOX) which may lead to a feed-forward stimulation of lipid peroxidation. Ebselen abolishes the multiple dangerous effects of PL-OOH by reducing them to the corresponding alcohols (PL-OH). In this manner, ebselen mimics the action of the selenocysteine enzyme PHGPx. The scheme also contains the biological role of the reaction of 15-LOX with biomembranes (for details see Schewe and Kühn, 1991). The symbols have the same meaning as in Fig. 3.

in particular of those known to produce free radicals and hence trigger oxidative stress. In many cases the cytoprotective action of ebselen and its metabolites is supposed to be due to their hydroperoxide-reducing capacity (Fig. 4). An exception could be the protection against paracetamol-induced toxicity; in that case the deleterious metabolite *N*-acetyl-*p*-benzoquinone imine was shown to be reduced by ebselen selenol (Li *et al.*, 1994). It is reasonable to assume that *in vivo* ebselen selenol is a more effective detoxifying reductant than thiols; thiols are however required for its formation from parent ebselen. In any case, ebselen may be used in the pharmacotherapy to lower the toxicity of drugs.

Actions in laboratory animals

A selection of experimental models that have been used to demonstrate the pharmacological efficacy of ebselen is listed in Table 5. Further examples have been reviewed in the paper of Parnham *et al.* (1991). These data substantiate the broad antiinflammatory potency of ebselen *in vivo*. It should be emphasised that in many studies ebselen proved to be superior to other non-steroidal antiinflammatory drugs such as indomethacin. In particular, unwanted side effects were not reported with ebselen. Thus, the application of ebselen is expected to improve the possibilities of the treatment of inflammatory diseases.

CONCLUDING REMARKS

Among the antioxidative drugs ebselen appears to be the most promising one. The favourable characteristics are mainly as follows:

—low toxicity and lack of adverse effects,
—metabolic stability due to several cycles of interconversions (see Fig. 1),
—biologic activities residing not only in ebselen itself but also in some of its metabolites,
—multiple molecular actions which include both detoxification of deleterious hydroperoxides and inhibition of enzyme activities as well as modulation of signal transduction processes and of cell–cell interactions,

Table 5. Antiinflammatory and protective actions of ebselen in laboratory animals

Experimental model	Species	Dosage (mg/kg)	Reference
Galactosamine/endotoxin-induced hepatitis	Mouse	600 p.o.	Wendel and Tiegs (1986)
Diet-induced pancreatitis	Mouse	100 s.c.	Niederau *et al.* (1991, 1992)
Experimental alveolitis and bronchiolitis	Rat	10 i.p.	Cotgreave *et al.* (1988)
Leukocyte and lymphocyte migration into inflamed joints	Rat	100 p.o.	Gao and Issekutz (1993a)
Adjuvant arthritis	Rat	100 p.o.	Gao and Issekutz (1993b, 1994)
Dietary gingivitis	Monkey	5% topical	Van Dyke *et al.* (1986)
Experimental allergic neuritis	Rat	10–100 p.o.	Hartung *et al.* (1986)
Hydrogen peroxide-induced monoarthritis	Mouse	50–100 p.o.	Schalwijk *et al.* (1986)
Hydrogen peroxide-induced foot pad oedema	Rat	50 p.o.	Griffith *et al.* (1992)
Cobra venom-induced oedema	Rat	56 p.o.	Leyck and Parnham (1990)
Ischaemic brain oedema	Rat	100 p.o.	Tanaka and Yamada (1989)
Ischaemic brain oedema	Cat	10 p.o.	Johshita *et al.* (1990)
Ischaemia-reperfusion injury of coronary artery	Dog	50 p.o.	Hoshida *et al.* (1994)
Ethanol or HCl-induced gastric mucosal injury	Rat	10–100 p.o.	Kurebayashi *et al.* (1989)
Ethanol-induced gastric mucosal injury	Mouse	30–100 p.o.	Tabuchi *et al.* (1995)
Gastric secretion and ulceration	Rat	30–100 intraduodenal	Tabuchi and Kurebayashi (1993)
Diclofenac-induced gastric intolerance	Rat	30–300 p.o.	Leyck and Parnham (1990)
Adriamycin-induced cardiotoxicity	Mouse	4 i.p.	Pritsos *et al.* (1992)

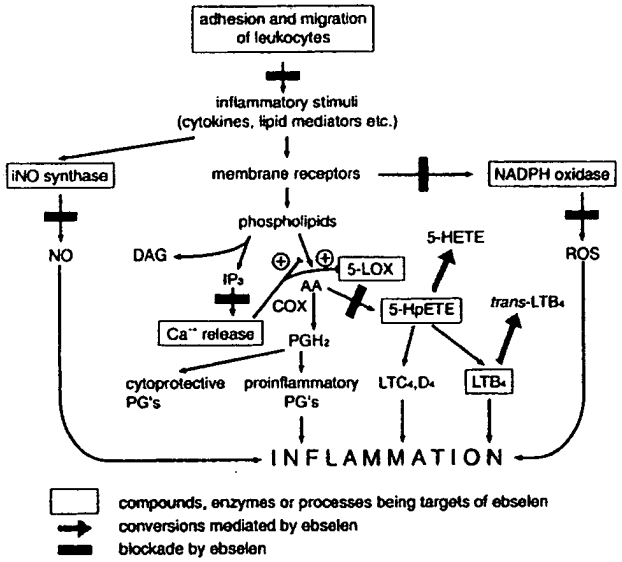

Fig. 5. Simplified scheme of the propagation of inflammation and the sites of antiinflammatory actions of ebselen. Abbreviations: COX, cyclooxygenase; LOX, lipoxygenase; DAG, diacylglycerol; H(p)ETE, hydro(pero)xyeicosatetraenoic acid; LT, leukotriene; PG, prostaglandin; IP_3, 1,4,5-inositol-*tris*-phosphate; ROS, reactive oxygen species; iNO synthase, inducible nitric oxide synthase.

—a unique antiinflammatory potential which is due to a set of multi-site actions (see Fig. 5) and which is not shared by any other anti-inflammatory drug,
—a pronounced cyto- and tissue-protective potential, in particular against oxidative stress (see Fig. 4),
—the capability of reducing drug toxicities,
—a prospective antiatherosclerotic potential (see Fig. 3).

While the antiinflammatory and cytoprotective activities of ebselen are well-established in a number of cellular systems and in studies with laboratory animals, publications on corresponding clinical trials are not yet available. Moreover, there is a backlog demand with respect to the research on the anti-atherosclerotic potential of ebselen. Finally, the biological activities of the ebselen species occurring in blood plasma need to be studied more exhaustively.

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REFERENCES

Akerboom T. P. M., Sies H. and Ziegler D. M. (1995) The oxidation of ebselen metabolites to thiol oxidants catalyzed by liver microsomes and perfused rat liver. *Arch. Biochem. Biophys.* **316**, 220–226.

Andersson C. M., Hallberg A., Linden M., Brattsand R., Moldéus P. and Cotgreave I. (1994) Antioxidant activity of some diarylselenides in biological systems. *Free Radic. Biol. Med.* **16**, 17–28.

Baldew G. S., Boymans A. P., Mol J. G. and Vermeulen N. P. (1992) The influence of ebselen on the toxicity of cisplatin in LLC-PK1 cells. *Biochem. Pharmacol.* **44**, 382–387.

Beil W., Staar U. and Sewing K. F. (1990) Interaction of the anti-inflammatory seleno-organic compound ebselen with acid secretion in isolated parietal cells and gastric H^+/K^+-ATPase. *Biochem. Pharmacol.* **40**, 1997–2003.

Brüne B., Diewald B. and Ullrich V. (1991) Ebselen affects calcium homeostasis in human platelets. *Biochem. Pharmacol.* **41**, 1805–1811.

Cembrzynska-Nowak M. and Inglot A. D. (1992) Human lymphoid target cells for the cytokine-inducing seleno-organic compounds. *Arch. Immunol. Ther. Exp. Warsz.* **40**, 235–240.

Christison J., Sies H. and Stocker R. (1994) Human blood cells support the reduction of LDL-associated cholesteryl ester hydroperoxides by albumin-bound ebselen. *Biochem. J.* **304**, 341–345.

Cotgreave I. A., Sandy M. S., Berggren M., Moldéus P. W. and Smith M. T. (1987) *N*-acetylcysteine and glutathione-dependent protective Effect of PZ 51 (ebselen) against diquat induced cytotoxicity in isolated hepatocytes. *Biochem. Pharmacol.* **36**, 2899–2904.

Cotgreave I. A., Johansson U., Westergren G., Moldéus P. W. and Brattsand R. (1988) The anti-inflammatory activity of ebselen but not thiols in experimental alveolitis and bronchiolitis. *Agents Actions* **24**, 313–319.

Cotgreave I. A., Duddy S. K., Kass G. E. N., Thompson D. and Moldéus P. (1989) Studies on the anti-inflammatory activity of ebselen. Ebselen interferes with granulocyte oxidative burst by dual inhibition of NADPH oxidase and protein kinase C? *Biochem. Pharmacol.* **38**, 649–656.

Cotgreave I. A., Morgenstern R., Engman L. and Ahokas J. (1992) Characterisation and quantitation of a selenol intermediate in the reaction of ebselen with thiols. *Chem.-Biol. Interactions* **84**, 69–76.

Czyrski J. A. and Inglot A. D. (1991) Mitogenic activity of selenoorganic compounds in human peripheral blood leukocytes. *Experientia* **47**, 95–97.

Dimmeler S., Brüne B. and Ullrich V. (1991) Ebselen prevents inositol (1,4,5)-trisphosphate binding to its receptor. *Biochem. Pharmacol.* **42**, 1151–1153.

Doroshow J. H. (1986) Prevention of doxorubicin-induced killing of MCF-7 human breast cancer cells by oxygen radical scavengers and iron chelating agents. *Biochem. Biophys. Res. Commun.* **135**, 330–335.

Engman L., Tunek A., Hallberg M. and Hallberg A. (1994) Catalytic effects of glutathione peroxidase mimetics on the thiol reduction of cytochrome *c*. *Chem.-Biol. Interactions* **93**, 129–137.

Fischer H. and Dereu N. (1987) Mechanism of the catalytic reduction of hydroperoxides by ebselen: a selenium-77 NMR study. *Bull. Soc. Chim. Belg.* **96**, 757–768.

Flohé L. (1989) The selenoprotein glutathione peroxidase. In *Glutathione: Chemical, Biochemical and Medical Aspects—Part A* (Edited by Dolphin D., Poulson R. and Avramovic O.), pp. 643–731. Wiley, New York.

Gao J.-X and Issekutz A. C. (1993a) The effect of ebselen on polymorphonuclear leukocyte and lymphocyte migration to inflammatory reactions in rats. *Immunopharmacology* **25**, 239–251.

Gao J.-X. and Issekutz A. C. (1993b) The effect of ebselen on polymorphonuclear leukocyte migration to joints in rats with adjuvant arthritis. *Int. J. Immunopharmac.* **15**, 793–802.

Gao J.-X. and Issekutz A. C. (1994) The effect of ebselen on T-lymphocyte migration to arthritic joints and dermal inflammatory reactions in the rat. *Int. J. Immunopharmac.* **16**, 279–287.

Geiger P. G., Lin F. and Girotti A. W. (1993) Selenoperoxidase-mediated cytoprotection against the damaging effects of *tert*-butyl hydroperoxide on leukemia cells. *Free Radic. Biol. Med.* **14**, 251–266.

Glass R. S., Farooqui F., Sabahi M. and Ehler K. W. (1989) Formation of thiocarbonyl compounds in the reaction of ebselen oxide with thiols. *J. Org. Chem.* **54**, 1092–1097.

Gower J. D., Lane N. J., Goddard J. G., Manek S., Ambrose I. J. and Green C. J. (1992) Ebselen. Antioxidant capacity in renal preservation. *Biochem. Pharmacol.* **43**, 2341–2348.

Griffiths H. R., Dowling E. J., Sahinoglu T., Blake D. R., Parnham M. and Lunec J. (1992) The selective protection afforded by ebselen against lipid peroxidation in an ROS-dependent model of inflammation. *Agents Actions* **36**, 107–111.

Gustafson D. L. and Pritsos C. A. (1991) Inhibition of mitomycin C's aerobic toxicity by the seleno-organic antioxidant PZ-51. *Cancer Chemother. Pharmacol.* **28**, 228–230.

Haenen G. R. M. M., de Rooij B. M., Vermeulen N. P. E. and Bast A. (1990) Mechanism of the reaction of ebselen with endogenous thiols. Dihydrolipoate is a better cofactor than glutathione in the peroxidase activity of ebselen. *Mol. Pharmacol.* **37**, 412–422.

Halliwell B. (1991) Drug antioxidant effects. A basis for drug selection? *Drugs* **42**, 569–605.

Harman A. W., Adamson G. M. and Shaw S. G. (1992) Protection from oxidative damage in mouse liver cells. *Toxicol. Lett.* **64–65**, 581–587.

Hartung H. P., Schäfer B., Heininger K. and Toyka K. V. (1986) Interference with arachidonic acid metabolism suppresses experimental allergic neuritis. *Ann. Neurol.* **20**, 168.

Hatchett R. J., Gryglewski R. J., Mlochowski J., Zembowicz A. and Radziszewski W. (1994) Carboxyebselen, a potent and selective inhibitor of endothelial nitric oxide synthase. *J. Physiol. Pharmacol.* **45**, 55–67.

Hattori R., Inoue R., Sase K., Eizawa H., Kosuga K., Aoyama T., Masayasu H., Kawai C., Sasayama S. and Yui Y. (1994) Preferential inhibition of inducible nitric oxide synthase by ebselen. *Eur. J. Pharmacol.* **267**, R1–R2.

Hoshida S., Kuzuya T., Nishida M., Yamashita N., Hori M., Kamada T. and Tada M. (1994) Ebselen protects against ischemia-reperfusion injury in a canine model of myocardial infarction. *Am. J. Physiol.* **267**, (*Heart Circ. Physiol.* **36**) H2342–H2347.

Hunt N. H., Cook E. P. and Fragonas J. C. (1991) Interference with oxidative processes inhibits proliferation of human peripheral blood lymphocytes and murine B-lymphocytes. *Int. J. Immunopharmacol.* **13**, 1019–1026.

Hurst J. S., Paterson C. A., Bhattacherjee P. and Pierce W. M. (1989) Effects of ebselen on arachidonate metabolism by ocular and non-ocular tissues. *Biochem. Pharmacol.* **38**, 3357–3363.

Hüther A. M., Zhang Y., Sauer A. and Parnham M. J. (1989) Antimalarial properties of ebselen. *Parasitol Res.* **75**, 353–360.

Inglot A. D., Zielinska-Jenczylik J., Piasecki E., Syper L. and Mlochowski J. (1990) Organoselenides as potential immunostimulants and inducers of interferon gamma and other cytokines in human peripheral blood leukocytes. *Experientia* **46**, 308–311.

Inglot A. D., Piasecki E., Zaczynska E. and Zielinska-Jenczylik J. (1992) Seleno-organic compounds induce interferon and tumor necrosis factor in human but not in rat or mouse lymphoid cells. *Arch. Immunol. Ther. Exp. Warsz.* **40**, 169–173.

Issekutz A. C. and Lopes N. (1992) Effect of ebselen on polymorphonuclear leukocyte adhesion to and migration through cytokine-activated vascular endothelium. *Int. J. Immunopharmacol.* **14**, 1383–1390.

Johshita H., Sasaki T., Matsui T., Hanamura T., Masayasu H., Asano T. and Takakura K. (1990) Effects of ebselen (PZ51) on ischemic brain oedema after focal ischemia in cats. *Acta Neurochir. Suppl.* **51**, 239–241.

Kagan V. E., Shvedova A., Serbinova E., Khan S., Swanson C., Powell R. and Packer L. (1992) Dihydrolipoic acid—a universal antioxidant both in the membrane and in the aqueous phase. *Biochem. Pharmacol.* **44**, 1637–1647.

Kamigata N., Iizuka H., Izuoka A. and Kobayashi M. (1986a) Photochemical reaction of 2-aryl-1,2-benzisoselenazol-3(2H)-ones. *Bull. Chem. Soc. Jap.* **59**, 2179–2183.

Kamigata N., Takata M., Matsuyama H. and Kobayashi M. (1986b) Novel ring opening reaction of 2-aryl-1,2-benzisoselenazol-3(2H)-one with thiols. *Heterocycles* **24**, 3027–3030.

Kamigata N., Takata M., Matsuyama H. and Kobayashi M. (1986c) Oxidation of thiols and sulfides by 2-aryl-1,2-benzisoselenazol-3(2H)-one 1-oxide. *Sulfur Lett.* **5**, 1–7.

Kodama K., Hirayama A. and Masayasu H. (1993) Inhibitor for restenosis after percutaneous coronary arterioplasty. *PCT Int. Appl.* WO 9313,762 (Cl. A61K31/165).

Kuhl P., Borbe H. O., Römer A, Fischer H. and Parnham M. J. (1985) Selective inhibition of leukotriene B4 formation by ebselen: a novel approach to antiinflammatory therapy. *Agents Actions* **17**, 366–367.

Kuhl P., Borbe H. O., Fischer H., Römer A. and Safayhi H. (1986) Ebselen reduces the formation of LTB4 in human and porcine leukocytes by isomerisation to its 5S,12R-6-transisomer. *Prostaglandins* **31**, 1029–1948.

Kühn H., Belkner J., Zaiss S., Fährenklemper T. and Wohlfeil S. (1994) Involvement of 15-lipoxygenase in early stages of atherogenesis. *J. Exp. Med.* **179**, 1903–1911.

Kühn-Velten N. and Sies H. (1989) Optical spectral studies of ebselen interaction with cytochrome *P*-450 of rat liver microsomes. *Biochem. Pharmacol.* **38**, 619–625.

Kurebayashi Y., Tabuchi Y. and Akasaki M. (1989) Gastric cytoprotection by ebselen against the injury induced by necrotizing agents in rats. *Arzneim.-Forsch./Drug Res.* **39**, 250–253.

Leurs R., Bast A. and Timmermann H. (1990) Ebselen inhibits contractile responses of guinea-pig parenchymal lung strips. *Eur. J. Pharmacol.* **179**, 193–199.

Leyck S. and Parnham M. J. (1990) Acute antiinflammatory and gastric effects of the seleno-organic compound ebselen. *Agents Actions* **30**, 426–431.

Li Q.-J., Bessems J. G. M., Commandeur J. N. M., Adams B. and Vermeulen N. P. E.. (1994) Mechanism of protection of ebselen against paracetamol-induced toxicity in rat hepatocytes. *Biochem. Pharmacol.* **48**, 1631–1640.

Lin F. and Girotti A. W. (1994) Cytoprotection against merocyanine 540-sensitized photoinactivation of the Na^+,K^+-adenosine triphosphatase in leukemia cells: glutathione and selenoperoxidase involvement. *Photochem. Photobiol.* **59**, 320–327.

Lin F., Geiger P. G. and Girotti A. W. (1992) Selenoperoxidase-mediated cytoprotection against merocyanine 540-sensitized photoperoxidation and photokilling of leukemia cells. *Cancer Res.* **52**, 5282–5290.

Maiorino M., Roveri A., Coassin M. and Ursini F. (1988) Kinetic mechanism and substrate specificity of glutathione peroxidase activity of ebselen (PZ 51). *Biochem. Pharmacol.* **37**, 2267–2271.

Maiorino M., Roveri A. and Ursini F. (1992) Antioxidant effect of ebselen (PZ 51): peroxidase mimetic activity on phospholipid and cholesterol hydroperoxides vs free radical scavenger activity. *Arch. Biochem. Biophys.* **295**, 404–409.

Miyazawa T., Suzuki T., Fujimoto K. and Kinoshita M. (1993) Elimination of plasma phosphatidylcholine hydroperoxide by a seleno-organic compound, ebselen. *J. Biochem. Tokyo* **114**, 588–591.

Moncada S. and Higgs A. (1993) The *L*-arginine-nitric oxide pathway. *N. Engl. J. Med.* **329**, 2002–2012.

Morgenstern R., Cotgreave I. A. and Engman L. (1992) Determination of the relative contributions of the diselenide and selenol forms of ebselen in the mechanism of its glutathione peroxidase-like activity. *Chem.-Biol. Interactions* **84**, 77–84.

Müller A., Cadenas E., Graf P. and Sies H. (1984) A novel biologically active seleno-organic compound. I. Glutathione peroxidase-like activity *in vitro* and antioxidant capacity of PZ 51 (ebselen). *Biochem. Pharmacol.* **33**, 3235–3239.

Müller A., Gabriel H. and Sies H. (1985) A novel biologically active selenoorganic compound. IV. Protective glutathione-dependent effect of PZ 51 (ebselen) against ADP-Fe induced lipid peroxidation in isolated hepatocytes. *Biochem. Pharmacol.* **34**, 1185–1189.

Nagi M. N., Laguna J. C., Cook L. and Cinti D. L. (1989) Disruption of rat hepatic microsomal electron transport chains by the selenium-containing anti-inflammatory agent ebselen. *Arch. Biochem. Biophys.* **269**, 264–271.

Narayanaswami V. and Sies H. (1990) Oxidative damage to mitochondria and protection by ebselen and other antioxidants. *Biochem. Pharmacol.* **40**, 1623–1629.

Niederau C., Ude K., Niederau M., Lüthen R., Strohmeyer G., Ferrell L. D. and Grendell J. H. (1991) Effects of the seleno-organic substance ebselen in two different models of acute pancreatitis. *Pancreas* **6**, 282–290.

Niederau C., Niederau M., Borchard F., Ude K., Luthen R., Strohmeyer G., Ferrell L. D. and Grendell J. H. (1992) Effects of antioxidants and free radical scavengers in three different models of acute pancreatitis. *Pancreas* **7**, 486–496.

Nikawa T., Schuch G., Wagner G. and Sies H. (1994a) Interaction of albumin-bound ebselen with rat liver glutathione S-transferase and microsomal proteins. *Biochem. Mol. Biol. Int.* **32**, 291–298.

Nikawa T., Schuch G., Wagner G. and Sies H. (1994b) Interaction of ebselen with glutathione S-transferase and papain *in vitro*. *Biochem. Pharmacol.* **47**, 1007–1012.

Noguchi N., Yoshida Y., Kaneda H., Yamamoto Y. and Niki E. (1992) Action of ebselen as an antioxidant against lipid peroxidation. *Biochem. Pharmacol.* **44**, 39–44.

Noguchi N., Gotoh N. and Niki E. (1994) Effects of ebselen and probucol on oxidative modifications of lipid and protein of low density lipoprotein induced by free radicals. *Biochim. Biophys. Acta* **1213**, 176–182.

Nomura H., Hakusui H. and Takegoshi T. (1989) Binding of ebselen to plasma protein. In *Selenium in Biology and Medicine* (Edited by Wendel A.) pp. 189–193, Springer, Heidelberg.

Ochi H., Morita I. and Murota S. (1992) Roles of glutathione and glutathione peroxidase in the protection against endothelial cell injury induced by 15-hydroperoxyeicosatetraenoic acid. *Arch. Biochem. Biophys.* **294**, 407–411.

Oshita M., Takei Y., Kawano S., Fusamoto H. and Kamada T. (1994) Protective effect of ebselen on constrictive hepatic vasculature: prevention of alcohol-induced effects on portal pressure in perfused livers. *J. Pharmacol. Exp. Ther.* **271**, 20–24.

Parnham M. J. and Graf E. (1987) Seleno-organic compounds and the therapy of hydroperoxide-linked pathological conditions. *Biochem. Pharmacol.* **36**, 3095–3102.

Parnham M. J. and Graf E. (1991) Pharmacology of synthetic organic selenium compounds. *Prog. Drug. Res.* **36**, 9–47.

Parnham M. J., Leyck S., Graf E., Dowling E. J. and Blake D. R. (1991) The pharmacology of ebselen. *Agents Actions* **32**, 4–9.

Patrick R. A., Peters P. A. and Issekutz A. C. (1993) Ebselen is a specific inhibitor of LTB_4-mediated migration of human neutrophils. *Agents Actions* **40**, 186–190.

Piasecki E., Inglot A. D., Zielinska-Jenczylik J., Mlochowski J. and Syper L. (1992) Simultaneous induction of interferon gamma and tumor necrosis factor alpha by different seleno-organic compounds in human peripheral blood leukocytes. *Arch. Immunol. Ther. Exp. Warsz.* **40**, 229–234.

Pritsos C. A., Sokoloff M. and Gustafson D. L. (1992) PZ-51 (ebselen) *in vivo* protection against adriamycin-induced mouse cardiac and hepatic lipid peroxidation and toxicity. *Biochem. Pharmacol.* **44**, 839–841.

Reich H. J. and Jasperse C. P. (1987) Organoselenium chemistry. Redox chemistry of selenocysteine model systems. *J. Am. Chem. Soc.* **109**, 5549–5551.

Roveri A., Maiorino M. and Ursini F. (1994) Enzymatic and immunological measurements of soluble and membrane-bound phospholipid-hydroperoxide glutathione peroxidase. *Meth. Enzymol.* **203**, 202–212.

Safayhi H., Tiegs G. and Wendel A. (1985) A novel biologically active seleno-organic compound. V. Inhibition by ebselen (PZ 51) of rat peritoneal neutrophil lipoxygenase. *Biochem. Pharmacol.* **34**, 2691–2694.

Sattler W., Maiorino M. and Stocker R. (1994) Reduction of HDL- and LDL-associated cholesterylester and phospholipid hydroperoxides by phospholipid hydroperoxide glutathione peroxidase and ebselen (PZ 51). *Arch. Biochem. Biophys.* **309**, 214–221.

Schalkwijk J., van den Berg W. B., van de Putte L. B. A. and Joosten L. A. B. (1986) An experimental model for hydrogen peroxide-induced tissue damage. Effects of a single inflammatory mediator on (peri)articular tissues. *Arthritis Rheum.* **29**, 532–538.

Schewe C., Schewe T. and Wendel A. (1994) Strong inhibition of mammalian lipoxygenases by the antiinflammatory seleno-organic compound ebselen in the absence of glutathione. *Biochem. Pharmacol.* **48**, 65–74.

Schewe T. and Kühn H. (1991) Do 15-lipoxygenases have a common biological role? *Trends Biochem. Sci.* **16**, 369–373.

Schöneich C., Narayanaswami V., Asmus K.-D. and Sies H. (1990) Reactivity of ebselen and related selenoorganic

compounds with 1,2-dichloroethane radical cations and halogenated peroxyl radicals. *Arch. Biochem. Biophys.* **282**, 18–25.

Scurlock R., Rougée M., Bensasson R. V., Evers M. and Dereu N. (1991) Deactivation of singlet molecular oxygen by organo-selenium compounds exhibiting glutathione peroxidase activity and by sulfur-containing homologs. *Photochem. Photobiol.* **54**, 733–736.

Serhan C. N. and Brezinski D. A. (1991) Lipoxin generation in human whole blood: monitoring by electron-capture NICI GC/MS. In *Prostaglandins, Leukotrienes, Lipoxins, and PAF. Mechanism of Action, Molecular Biology, and Clinical Applications* (Edited by Bailey J. M.), pp. 421–430. Plenum Press, New York.

Sies H. (1989) Metabolism and disposition of ebselen. In *Selenium in Biology and Medicine* (Edited by Wendel A.), pp. 153–162. Springer, Heidelberg.

Sies H. (1993) Ebselen, a selenoorganic compound as glutathione peroxidase mimic. *Free Radic. Biol. Med.* **14**, 313–323.

Sies H. (1994) Ebselen: a glutathione peroxidase mimic. *Meth. Enzymol.* **234**, 476–482.

Steinberg D., Parthasarathy S., Carew T. E., Khoo J. C. and Witztum J. (1989) Beyond cholesterol. Modification of low density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320**, 915–924.

Tabuchi Y. and Kurebayashi Y. (1993) Antisecretory and antiulcer effects of ebselen, a seleno-organic compound, in rats. *Jap. J. Pharmacol.* **61**, 255–257.

Tabuchi Y., Ogasawara T. and Furuhama K. (1994) Mechanism of the inhibition of hog gastric H^+, K^+-ATPase by the seleno-organic compound ebselen. *Arzneim.Forsch. —Drug Res.* **44**, 51–54.

Tabuchi Y., Sugiyama N., Horiuchi T., Furusawa M. and Furuhama K. (1995) Ebselen, a seleno-organic compound, protects against ethanol-induced murine gastric mucosal injury in both *in vivo* and *in vitro* systems. *Eur. J. Pharmacol.* **272**, 195–201.

Tanaka J. and Yamada F. (1989) Ebselen (PZ-51) inhibits the formation of brain edema. In *Selenium in Biology and Medicine* (Edited by Wendel A.), pp. 173–176, Springer, Heidelberg.

Thomas C. E. and Jackson R. L. (1991) Lipid hydroperoxide involvement in copper-dependent and independent oxidation of low density lipoproteins. *J. Pharmacol. Exp. Ther.* **256**, 1182–1188.

Thomas J. P., Geiger P. G., Girotti A. W. (1993) Lethal damage to endothelial cells by oxidized low density lipoprotein: role of selenoperoxidases in cytoprotection against lipid hydroperoxide- and iron-mediated reactions. *J. Lipid. Res.* **34**,479–490.

Van Dyke T. E., Braswell L. and Offenbacher S. (1986) Inhibition of gingivitis by topical application of ebselen and rosmarinic acid. *Agents Actions* **19**, 376–377.

Wagner G. Schuch G., Akerboom T. P. M. and Sies H. (1994) Transport of ebselen in plasma and its transfer to binding sites in the hepatocyte. *Biochem. Pharmacol.* **48**, 1137–1144.

Wang J.-F., Komarov P., Sies H. and de Groot H. (1992) Inhibition of superoxide and nitric oxide release and protection from reoxygenation injury by ebselen in rat Kupffer cells. *Hepatology* **15**, 1112–1116.

Weitzel F. and Wendel A. (1993) Selenoenzymes regulate the activity of leukocyte 5-lipoxygenase via the peroxide tone. *J. Biol. Chem.* **268**, 6288–6292.

Weitzel F., Ursini F. and Wendel A. (1990) Phospholipid hydroperoxide glutathione peroxidase in various mouse organs during selenium deficiency and repletion. *Biochim. Biophys. Acta* **1036**, 88–94.

Wendel A. and Tiegs G. (1986) A novel biologically active seleno-organic compound. VI. Protection by ebselen (PZ 51) against galactosamine/endotoxin-induced hepatitis in mice. *Biochem. Pharmacol.* **35**, 2115–2118.

Wendel A., Fausel M., Safayhi H., Tiegs G. and Otter R. (1984) A novel biologically active seleno-organic compound. II. Activity of PZ 51 in relation to glutathione peroxidase. *Biochem. Pharmacol.* **33**, 3241–3245.

Wendel A., Otter R. and Tiegs G. (1986) Inhibition by ebselen of microsomal NADPH-cytochrome *P* 450-reductase *in vitro* but not *in vivo*. *Biochem. Pharmacol.* **35**, 2995–2997.

Ylä-Herttuala S., Rosenfeld M. E., Parthasarathy S., Glass C. K., Sigal E., Witztum J. L. and Steinberg D. (1990) Colocalisation of 15-lipoxygenase mRNA and protein with eptopes of oxidized low density lipoprotein in macrophage-rich areas of atherosclerotic lesions. *Proc. Natl. Acad. Sci.* **87**, 6959–6952.

Yoshikawa T., Naito Y. and Kondo M. (1993) Antioxidant therapy in digestive diseases. *J. Nutr. Sci. Vitaminol. Tokyo* **39 Suppl.**, S35–S41.

Zembowicz A., Hatchett R. J., Radziszewski W. and Gryglewski R. J. (1993) Inhibition of endothelial nitric oxide synthase by ebselen. Prevention by thiols suggests the inactivation by ebselen of a critical thiol essential for the catalytic activity of nitric oxide synthase. *J. Pharmacol. Exp. Ther.* **267**, 1112–1118.

Zhang Y.-Y., Hamberg M., Radmark O. and Samuelsson B. (1994) Stabilization of purified human 5-lipoxygenase with glutathione peroxidase and superoxide dismutase. *Anal. Biochem.* **220**, 28–35.

Ziegler D. M., Graf P., Poulsen L. L., Stahl W. and Sies H. (1992) NADPH-dependent oxidation of reduced ebselen, 2-selenylbenzanilide, and of 2-(methylseleno)benzanilide catalyzed by pig liver flavin-containing monooxygenase. *Chem. Res. Toxicol.* **5**, 163–166.

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Diaryl chalcogenides as selective inhibitors of thioredoxin reductase and potential antitumor agents.

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Thioredoxin reductase is a selenocysteine containing flavoenzyme that catalyzes the NADPH dependent reduction of the redox protein thioredoxin. Thioredoxin is over-expressed by a number of human tumors. Experimental studies have shown that thioredoxin is responsible for the growth and transformed phenotype of some human cancer cells. Thus, thioredoxin reductase presents an attractive target for anticancer drug development to regulate the activity of the thioredoxin system. We have examined a series of 12 organoselenium compounds and 16 organotellurium compounds, mostly of the diaryl chalcogenide type, as inhibitors of human thioredoxin reductase and have investigated the cytotoxicity and antitumor activity of some of the compounds. The organoselenium compound Ebselen was found to be a competitive inhibitor of human thioredoxin reductase (Ki 2.8 microM), while a number of organotellurium compounds were found to be noncompetitive inhibitors (Kis 2.3 to 35.2 microM). Human glutathione reductase was not appreciably inhibited by any of the compounds, except for one dinitro organotellurium compound that caused inhibition with an IC50 of 0.5 microM and an over 20-fold selectivity compared to thioredoxin reductase. The compounds inhibited the growth of human cancer cells in culture with IC50s as low as 2 microM Some organotellurium compounds when administered daily by intraperitoneal injection to mice caused up to 50% inhibition of the growth of MCF-7 human breast cancer xenografts but the relative insolubility of the compounds was a limiting factor in their use.

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